Organic Substances and Sediments in Water

Volume 3

Biological

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Robert A. Baker
Editor

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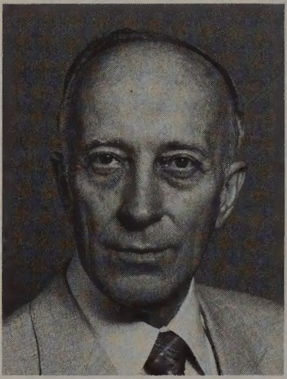
To
Peggy Baker
for her continued understanding and encouragement



Robert A. Baker is affiliated with the National Research Program, Water Resources Division of the U.S. Geological Survey. He holds a BChE from North Carolina State University, MChE and MS from Villanova University, and a D.Sc. from the Graduate School of Public Health, University of Pittsburgh. Dr. Baker's professional career has involved research, consultation, and management related to environmental science and engineering problems. He is active in professional societies and has authored over 70 books, patents, and papers.

Preface

Water resources managers, regulators, and researchers require definitive information that describes the highly correlated, interdisciplinary factors that influence fate and transport of water contaminants. Not unexpectedly, evolving questions stay ahead of advances in scientific and engineering developments. One of the most important and significant aspects currently being intensely investigated is the role of particulates and sediments in contaminant behavior. This three-volume compilation documents the proceedings of a symposium dedicated to the subject of organic substances and sediments in water. Stress was placed on the organic substances because so many of the anthropogenic contaminants which pose potential problems at all trophic levels are organic in nature.

The symposium program from which the proceedings derive included critical reviews which describe the state-of-the-science, and often identify major needs. This should be especially valuable to the reader, regardless of individual interest. As in any symposium proceedings, topics are treated with varying depth. However, coverage over the interdisciplinary subject is reasonably complete.

The first volume delves into the roles of humic substances and soils-sediments in the sorption and mobility of contaminants. Both regimes are introduced by comprehensive review papers, and both reviews are followed by papers that treat specific topics in depth.

The second volume combines papers that summarize various processes involved in contaminant fate and transport as well as analytical developments. The processes section has been divided into aquatic particle-organic chemical interaction (characterization and contaminant geochemistry); fate and transport; and interfacial and organic-inorganic processes. The processes and analytical sections present theoretical as well as case study developments.

The third volume is devoted to biological processes. It begins with a state-of-the-science summary which incorporates references to the other papers deriving from the symposium. The papers are divided under subheadings: integrating chemistry and toxicology of sediment-water interactions; uptake and accumulation (bioavailability and bioaccumulation); biodegradation (aerobic dechlorinations and co-metabolism).

This compilation extends over the broad interdisciplinary subject of organic substances and sediments in water. It should prove valuable to experienced scientists as well as those making initial inquiries.

Acknowledgments

An American Chemical Society symposium on the subject "Contaminants and Sediments" was held in Honolulu, April 1–6, 1979. A two-volume publication of the same title was published by Ann Arbor Science in 1980. These publications have frequently been cited in the literature. Several years ago colleagues suggested that the writer consider organization of another symposium to foster technology transfer and to update the proceedings of the previous state-of-the-science summary. This led to the symposium "Organic Substances and Sediments in Water" held at the American Chemical Society Meeting in Boston, April 22–27, 1990. The symposium emphasized organic substances and the complex processes effecting their fate and transport, particularly as these occur at the interface of suspended and fixed surfaces. Interdisciplinary contributions were solicited and development of topical sessions shared with recognized experts. These were: V. D. Adams, Tennessee Technical University; D. Armstrong, University of Wisconsin; S.A. Boyd, Michigan State University; C.T. Chiou, U.S. Geological Survey; B. Dempsey, Pennsylvania State University; B.J. Eadie, Great Lakes Environmental Research Laboratories; S.J. Eisenreich, University of Minnesota; P.F. Landrum, Great Lakes Environmental Research Laboratory; J. Leenheer, U.S. Geological Survey; R.L. Malcolm, U.S. Geological Survey; J.F. McCarthy, Oak Ridge National Laboratory; A.V. Palumbo, Oak Ridge National Laboratory; and A. Stone, Johns Hopkins University. Their dedication and cooperation was of the finest from onset through final manuscript peer review.

In addition to North American participants, scientists and engineers from other continents contributed. Five invited European scientists were: Jacques Buffle, University of Geneva, Switzerland; Hans Borén, Linköping University, Sweden; Egil Gjessing, Norwegian Institute for Water Research, Oslo, Norway; Jussi Kukkonen, University of Joensuu, Finland; and Paolo Sequi, Istituto D. Chimica Agraria, Bologna, Italy. Their perceptions and comments were as valuable as their technical contributions. A grant from the U.S. Environmental Protection Agency provided travel support for the invited speakers. Louis Swaby, Office of Exploratory Research, Washington, DC, and Wayne Garrison, Environmental Research Laboratory, Athens, Georgia provided program development assistance and liaison.

Chemical sciences are often an integral aspect of scientific and engineering processes perceived as nonchemical in nature. To improve knowledge of such situations and to facilitate communication among interdisciplinary contributions, the American Chemical Society, through its Committee on Science, has established a Pedagogical Symposium program. These tutorial symposia typically offer overview and research presentations by acknowledged experts in related fields. A competitive proposal to conduct a pedagogical symposium on

the same subject as the research symposium was awarded by the Committee on Science. The tutorial was held on April 24, 1990. The lecturers were: E.J. Bouwer, Johns Hopkins University; D.M.D. Toro, Manhattan College; J.W. Farrington, University of Massachusetts; I. Knight, University of Maryland; J.R. Pratt, Pennsylvania State University; R.E. Speece, Vanderbilt University; and J.A. Symons, University of Houston. Their presentations dramatically demonstrated the interdependence of various scientific and engineering processes as well as the benefits of interdisciplinary technology transfer. Drs. Pratt, Speece, and Knight contributed papers to these proceedings.

Financial support for the pedagogical symposium was from the Committee on Science and from the Environmental Chemistry Division of the American Chemical Society. The research symposium and the pedagogical symposium were held under the auspices of the Environmental Chemistry Division. Encouragement and support of the officers and members of these organizational units is gratefully acknowledged.

The endeavor would have been of no avail without the contribution of the scientists and engineers whose manuscripts are contained in these proceedings. The editor appreciates their willingness to share knowledge.

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PART I

INTRODUCTION

CHAPTER 1

Organic Contaminants in Sediments: Biological Processes

John F. McCarthy, Peter F. Landrum, and Anthony V. Palumbo

INTRODUCTION

A wide variety of organic contaminants enter the environment through myriad sources, including inputs from industrial or municipal effluents, ocean dumping of wastes, terrestrial runoff, and atmospheric deposition. Many aspects of the physical and chemical processes affecting the transport and fate of contaminants within sediment are addressed in other chapters of this book on organic substances and sediments in water. This chapter focuses on the role of biological processes in sediments, either in the uptake of toxic chemicals by biota, the redistribution of sediments and sediment-bound contaminants by burrowing organisms, or microbial metabolism as a mechanism for remediating existing pollution. Biological processes are, of course, central to issues of environmental contamination. Our concern about toxic chemicals in the environment stems not from a mere academic interest in their chemistry, but rather a more anthropocentric concern for the adverse health and ecological effects of these compounds. In this volume, Pratt provides a pedagogical review of environmental toxicology, describing the current approaches to evaluating the effect of hazardous chemicals on ecological processes.[1]

Two major biological processes relevant to the toxic effects of sediment-associated contaminants are: bioaccumulation of contaminants from sediments (including the role of bioturbation in redistribution of contaminants to the bioactive zone), and biodegradation of toxic compounds within aquatic and aquifer sediments. In this chapter, we provide an overview of current understanding and major research directions being pursued in these areas. In this overview, the contributions of authors of subsequent chapters are highlighted.

BIOAVAILABILITY OF SEDIMENT-ASSOCIATED CONTAMINANTS

Many of the contaminants of ecological interest are compounds possessing characteristics that make them resistant to degradation and cause them to sorb strongly to particles. The movement of these particles to the sediments is the main route for the clearance of these persistent contaminants from the water column. These particles are subsequently transported to depositional areas in the particular water body by the existing current regime. Such movement results in the concentration of the contaminants primarily in the low-energy depositional regions of water bodies. These deposits can result in extremely high concentrations of contaminants in localized regions. Some of the more common areas for such concentration include harbors, which are generally areas of historic industrial contaminant discharge as well as areas of deposition. Further, by their construction or natural features, harbors are low-energy environments and often have very high rates of deposition. Such harbor areas have the highest levels of sediment-associated contaminants. In addition to their high levels of contamination, harbors are also areas requiring frequent dredging for navigational purposes. This dredging can result in the resuspension of buried contaminants.

In the context of dredging, the issue of contaminated sediments has been recognized for many years, and regulations have been established to prohibit the open-water disposal of contaminated materials dredged from harbors because of their potential impact on the biological community.[2] The significance of in-place or sediment-associated contaminants as sources of problems outside the dredging arena has been slower to be recognized. However, with the improvements of water quality through controls on discharges, these in-place contaminants are currently recognized as important contaminant sources equal to nonpoint runoff and atmospheric deposition.

In addition to harbor areas, natural depositional basins may also collect sufficient contaminant concentrations to result in alteration of benthic communities, as has been observed in the Great Lakes.[3] While most of the observed effects of contaminated sediments have been found in harbors and near shore areas, even the open-water depositional basins of the Great Lakes show sufficient contaminant concentrations to elicit effects on oligochaetes in laboratory bioassays.[4] From a regulatory perspective, it is often important to be able to determine the contribution of different sources of anthropogenic pollutants to bioaccumulation. In this volume, Sherblom and Eganhouse evaluate the use of source-specific marker compounds detected in biota to document and characterize the impact of municipal wastewaters on tissue burdens of specific contaminants.[5]

In addition to recognizing the impact of contaminated sediments on particular benthic species, sediment-associated contaminants have also been recognized as a potentially significant food chain contaminant source. This general recognition of the importance of contaminated sediments has highlighted the need to establish sediment quality criteria. These criteria must protect both the indigenous benthic species that are directly exposed to sediment-associated contaminants and account for the accumulation of the contaminants through the food chain. Development of such criteria requires a fundamental understanding of the bioavailability of sediment-associated contaminants and the factors that control this bioavailability.

In order to understand bioavailability, a definition and a measure must be available so that comparisons can be made. Bioavailability of sediment-associated contaminants can be defined as "the fraction of the total contaminant in the interstitial water and on the sediment particles that is available for bioaccumulation,"[6] where *bioaccumulation* is the accumulation of a contaminant via all routes available to the organism. Chemical measures of contaminant concentration in sediment do not always reflect the bioavailable fraction of the sediment-associated contaminants;[7,8] therefore, a simple measurement of sediment residue is insufficient to describe the contaminant concentration to which biota are truly exposed. Two approaches have been described in the literature to describe the bioavailability of sediment-associated contaminants: (1) a comparison of the contaminant concentration in the organism and the sediment at steady state,[9–13] and (2) measurement of the uptake clearance of sediment-associated contaminants.[7,14–19] In spite of having two biological approaches available, no chemical approach is available to define the bioavailable fraction of sediment-bound residues. Thus, better approaches for describing the bioavailable fraction of sediments are needed.

The main factors that appear to affect bioavailability can be divided into two major areas: factors that alter the partitioning of contaminants to sediments, and biological factors that alter the exposure and accumulation of the contaminants.[20–23] In all cases, the physicochemical characteristics of the organic contaminant, primarily the compound's water solubility and hydrophobicity, are the major factors for determining its bioavailability. A complicating factor in any physicochemical assessment of bioavailability is the role of natural "dissolved" (or colloidal) organic matter (DOM) in sorbing hydrophobic organic contaminants and metals.[24] In general, organic contaminants sorbed to DOM are unavailable for uptake by biota.[14,25–27] However, predictions of the fraction of truly dissolved (i.e., bioavailable) pollutant in bulk or interstitial water is hampered by poorly understood variability in the contaminant binding capacity of DOM from different natural waters.[28–29] In this volume, Evans demonstrates that the DOM present in water from one Canadian lake has little effect on bioaccumulation of a polychlorinated biphenyl (PCB).[30] In contrast, Kukkonen et al. (also in this volume) examine a range of natural waters and demonstrate that the chemical and physical properties of DOM influence the extent of binding of polycyclic aromatic hydrocarbon (PAH) versus PCBs, but that the reduction in bioavailability remains correlated with physicochemical association of the contaminant with the natural organic macromolecule.[31] Södergren (also this volume) proposes an innovative monitoring tool to empirically estimate the bioavailable fraction of a contaminant in a natural system; the approach is based on the use of solvent-filled dialysis bags to mimic bioaccumulation of organic contaminants by lipid pools of aquatic organisms.[32]

Accurate estimation of physicochemical partitioning of contaminants is not, by itself, sufficient to predict accumulation of environmental contaminants. Bioavailability of the sediment-associated contaminants needs to be understood as a complex interaction between physical, chemical, and biological characteristics that change with site, compound, and species of interest, thus making it difficult to understand and predict the bioavailability without detailed, site-specific information. Lee (this volume) illustrates this point by demonstrating the complicating influences of feeding behavior, burrowing characteristics, contact time of pollutants with sediment, and nonequilibrium sorption processes on bioaccumulation of organic contaminants in benthic invertebrates.[19]

An important, but poorly understood, aspect of contaminant accumulation from sediment is the relative role of water versus sediment particles as the source of exposure. The uptake of contaminants by sediment-associated organisms has generally been reported as primarily via the interstitial water; however, more recent studies suggest that ingestion of contaminated particles may play as large a role—or even a larger role—in bioaccumulation of sediment-associated contaminants.[7,33] Establishing which of the routes of accumulation are most important for these contaminants—both for specific benthos and for organisms strongly coupled to the benthos through food chain transport—will dictate the approach required for establishing sediment quality criteria. There are currently several approaches for the establishment of sediment quality criteria, including, but not limited to, sediment quality triad, apparent effects threshold, screening level concentration, equilibrium partitioning approach, and spiked sediment bioassays. In terms of effects on the benthos, several of the approaches give very similar estimates of the level of sediment-associated contaminants producing effects.[34] In spite of the similarity of the results from the above comparison, the science is not sufficiently established that one method for establishing criteria can be selected over another. Given the weaknesses in our current understanding, the use and comparison of several of the available methods may be the best approach for making decisions about the extent and significance of sediment contamination.

Even with several methods available for estimating an effects level for specific contaminants, there are limitations to the types of situations that can be addressed. There is no method that can establish the appropriate level of contaminants where the exposure occurs in mixtures, the usual situation in sediment exposures. Further, most of the bioassays currently in use for contaminated sediments address acute and, in some cases, subacute end points. There is, in general, an absence of bioassays available for measuring chronic effects. Finally, none of the criteria approaches consider the effects of food chain transfer and its impact on higher trophic level species.

An alternate approach to evaluating the significance of sediment exposures is the measurement of biological markers in macroinvertebrates and fish collected from contaminated areas. In this approach, it is the biochemical or physiological expression of contaminant exposure and effect (e.g., genotoxic damage, induction of detoxication systems, or histological lesions) that is assayed, rather than the level of individual contaminants in either the sediment or the organism.[35] Biomarkers have the advantage that the complexities of physicochemical partitioning, bioaccumulation, and pharmacodynamics are bypassed, and the end points of most direct concern to animal health are probed explicitly. Long and Buchman have compared and found reasonable agreement between the results of biomarker-based biomonitoring of San Francisco Bay with several more traditional end points such as toxicity tests and concentrations of chemicals in sediments.[36] While biomarker responses measured in organisms from sites with contaminated sediments offer the promise of providing an integrated assessment of the biological significance of exposure, the approach is still under development and requires more research before it can be used as a reliable indicator of sediment quality.

In the above discussion, the concentrations of contaminants in the sediment were assumed to be constant. However, both physical and biological processes can affect the amount of a chemical in the sediment. One of the major factors (in addition to the biological and partitioning factors considered above) that will modify exposure is alteration of the sediment concentration through removal of contaminated sediments from the bioactive layer. The influx of fresh sediment will bury and, to some extent, dilute the contaminated sediment in the bioactive layer, particularly if remedial changes have reduced the concentration on incoming particles. This burial can, however, be modified through bioturbation by benthos that feed in a conveyor-belt fashion. Such bioturbation processes will result in the transport of contaminant-laden particles to the top of the bioactive zone.[37,38] These bioturbing processes extend the duration of exposure of benthic organisms to incoming particle-associated contaminants. Such actions, in conjunction with changes in the depositional regimes, may actually result in the reappearance of contaminants into the bioactive layers of sediments.

A productive approach to integrating the range of physical, chemical, and biological processes that can affect bioaccumulation from sediments involves mathematical modeling of multiply-interacting processes. Mackay et al. (this volume) illustrate the useful insights that can be obtained with "environmental video games" based on a mass balance model of pollutant fate, transport, and bioaccumulation.[39] Young et al. (also this volume) provide an example of field observations of DDE and PCB accumulation from marine sediments that are encouragingly consistent with the fugacity models.[40]

The principal objective of this portion of the symposium is to highlight and illustrate the complex interactions that dictate the total exposure of the biota to sediment-associated contaminants and to indicate gaps in our understanding that limit capabilities to predict the results of such exposures. Even with the number of papers on the interaction of biota with sediment-associated contaminants increasing rapidly, this field is very young and of such complexity that a complete understanding will require many years of effort. As a result, complete understanding of this field will not occur rapidly and interim approaches will be required for assessing and controlling contaminated sediments.

BIODEGRADATION OF SEDIMENT-ASSOCIATED CONTAMINANTS

While our focus on contaminants in sediments may be motivated by our concern about their toxic effects on biological systems, it is one of the symmetries of nature that biological processes also act to alleviate these concerns by catalyzing the degradation of contaminants. While the degradative capabilities of microorganisms have long been recognized, they have more recently become the focal point for environmental initiatives aimed at restoring contaminated areas. Attempts to remove or isolate contamination through physical and chemical techniques are expensive and often ineffective. It has become clear that in situ bioremediation offers the best practical hope for removing contaminants in natural systems.

Although many organic contaminants that associate with sediments have proven to be highly resistant to aerobic biodegradation, new approaches and understandings are promising increased potential for the biodegradation of many of these contaminants. The purpose of this section is not to extensively review biodegradation of sediment-associated contaminants, but rather to highlight productive and promising research areas associated with biodegradation and to place the papers in this section in the context of current research. Increasing attention to anaerobic degradation, particularly anaerobic dechlorinations,[41,42] and cometabolism has led to a greater appreciation of the degradability of organic compounds by mechanisms not commonly examined. Also, a wider range of compounds have also proven to be degradable by aerobic mechanisms (e.g., PCBs). Often the expression of these degradative capabilities in nature is limited, but genetic engineering offers the potential for increasing the expression of degradative genes and for providing tools for the examination of biodegradation in sediments. Knight and Colwell (in this volume) provide an overview of both the theory and practice of molecular genetics and recombinant DNA technology.[43]

The search for understanding of the degradation of sediment-associated organic compounds has been pursued in many types of sedimentary environments. Exciting research on degradation of PCBs in aquatic sediments has been complemented by new ideas and approaches being developed in examinations of aquifer sediments and in unsaturated terrestrial sediments. The most significant work on biodegradation through anaerobic dechlorination has focused on the fate of PCBs in aquatic sediments, most of which are anaerobic. On the other hand, many of the interesting new findings related to both traditional aerobic degradation and to cometabolic mechanisms come from work on unsaturated terrestrial sediments or on oxygenated aquifer sediment and groundwater.

Traditionally, many types of contaminants that have been considered to be resistant to biodegradation—ranging from relatively simple chlorinated ethenes (such as trichloroethylene), to much larger, more complex chlorinated compounds (such as PCBs), and large, complex nonchlorinated organic compounds (such as the PAHs). Different classes of these compounds are likely to be found in different sediment settings, and this influences the type of degradative processes that are important in the fate of these compounds. The higher-molecular-weight, nonpolar classes are relatively insoluble in water. Thus, they can be found in aquatic sediments which are likely to be anaerobic, in unsaturated soils where they are likely to be exposed to aerobic conditions, and in groundwater sediments where the conditions may either be aerobic or anaerobic. The lower-molecular-weight, polar compounds are likely only to be found in the terrestrial systems, such as the surface soils, aquifer sediment, and groundwater environments. These compounds would probably not become associated with aquatic sediments due to their relatively high aqueous solubility. However, they may be found in detectable quantities in aquifer sediments due to limited water flow through these sediments. The examination of these varied environments is leading to new approaches to biodegradation, including the linking of anaerobic-aerobic systems for degradation of chlorinated compounds.[44]

There are both broad similarities in the degradation of compounds in terrestrial and aquatic sediments and significant differences. One similarity is the issue of the bioavailability of the high-molecular-weight, nonpolar compounds. In aquatic sediments, unsaturated terrestrial sediments, and aquifer sediments, these compounds will tend to be strongly sorbed to particles. In this state they are likely to more resistant to biological degradation simply due to the inability of the bacteria to access this material. Due to the similarities among the sediment systems, comparisons of processes and rates of bacterial activity in aquatic and aquifer sediments can be useful in obtaining insights into microbial function in these environments.[45] A significant difference between the aquatic sediments and aquifer sediments is the presence of macroinvertebrates and other fauna in the aquatic sediments. Bioturbation and other effects can increase biodegradation rates of organic contaminants on sediments.[46,47] Also, the presence of exotic natural compounds, such as halophenols, may stimulate populations able to degrade similar organic contaminants, thus serving as a source of bacteria for biodegradation of a much broader array of contaminants.[48]

Anaerobic Dechlorinations

Intense research on the reductive dechlorinations of PCBs was prompted by the observation of dechlorination of PCBs in aquatic sediments.[42,49,50] Analysis of other sediments by Lake et al. (this volume) has expanded the range of sediments in which dechlorinations of PCBs have been observed.[51] Laboratory demonstrations of dechlorination of PCBs soon followed.[52] Questen et al. have found that *meta* and *para* chlorines were removed during dechlorination and that dechlorination was slower and less effective with increasing chlorination.[44] They also found that sediment samples from different sites contained microorganisms with different specificities for PCB dechlorination.

Chlorinated ethylenes (CEs) are also degraded via anaerobic dechlorination. Bouwer et al. have observed anaerobic degradation of perchloroethylene (PCE), trichloroethylene (TCE), and dichloroethylene (DCE) by dechlorination mechanisms and concluded that methanogenesis was the principal biochemical reaction responsible for transforming TCE and PCE.[53,54] Fathepure et al. have demonstrated that a pure methanogenic culture (*Methanosarcina* sp. DCM) degraded TCE and PCE and proposed that PCE was reduced by the same electron carrier that would normally reduce carbon dioxide to methane.[55,56] Freedman and Gossett have shown that numerous chlorinated ethenes may be dechlorinated by a mixed methanogenic culture.[57] Although anaerobic dechlorination is a slow process, it is the only known mechanism for PCE degradation. Unfortunately, PCE or TCE dechlorination often yields vinyl chloride, which is considered to be one of the most carcinogenic compounds.[58]

Dechlorinations of other compounds, such as chloroaromatic compounds, pesticides, and herbicides, have also been a recent focus of study,[41] and much of that work indicates that dehalogenation does not occur with sulfate as an elector acceptor.[59] Dehalogenation may be inhibited in groundwater aquifer sediments by the presence of sulfate,[60] and similar results have been reported for aquatic sediments.[61] Suflita et al. (this volume) examine both the conditions promoting anaerobic biotransformation of herbicides (finding dehalogenation under methanogenic conditions) and the pathways for the degradation.[62] The relationship between sediment/water properties and the reductive dechlorination of various other compounds is addressed by Hale et al. (also in this volume), who have related reductive dechlorination of dichlorophenols to characteristics such as pH, nitrate and sulfate concentrations, and redox potential.[63]

Cometabolism

The terms *cometabolism, fortuitous metabolism,* and *cooxidation* are often used to describe the same process, and much debate is centered on the use of these terms. In aerobic degradation, the bacteria obtain carbon and energy from oxidation of the contaminants, whereas with cometabolism this benefit to the bacteria does not occur. Dalton and Stirling define cometabolism as having four features:[64]

1. A cometabolite does not support growth.
2. The products accumulate stoichiometrically.

3. Transformation is associated with increased oxygen uptake.
4. Cometabolism entails adventitious utilization of existing enzyme systems.

Most cometabolic metabolism has been associated with the activity of various mono- and dioxygenase enzymes. Some of the primary groups of organisms associated with cometabolic metabolism are methane oxidizers, toluene degraders, and ammonia oxidizers. Mono- and dioxygenase enzymes in other types of bacteria have also been implicated in the cometabolic degradation.

The key to cometabolism is the broad substrate specificity of the oxygenase enzymes. Perhaps the best-studied enzyme involved in cometabolism is methane monooxygenase (MMO). The interest in MMO was largely prompted by the work of Wilson and Wilson on TCE degradation.[65] Since then, all CEs except PCE have been reported to be degraded by methanotrophic mixed cultures.[66–70] This MMO enzyme catalyzes the transformation of methane to methanol in the presence of nicotinamide-adenine-dinucleotide (NADH) and oxygen. MMO has been found to have a broad substrate range, which in addition to methane and the CEs includes benzene, bicyclohexyl, butadiene, chlorophenol, cresol, ethylbenzene, phenol, ethene, propene, pyridine, styrene, toluene, and xylene,[71] as well as carbon monoxide[72,73] and ammonia.[74] The details of the pathways and kinetics, as well as the effect of groundwater chemistry on CE degradation, have been examined in a number of studies using pure cultures.[75–77] Palumbo et al. (in this volume) describe the inhibitory effect of ammonia as well as the effect of other groundwater components on TCE degradation by the methanotrophs.[78]

The utility of methanotrophs for in situ degradation of CEs in aquifer sediments has been examined in simulated and actual aquifer systems. Moore et al. spiked a methane-stimulated aquifer with *trans*-1,2-DCE (1 mg/L) and found that 80% of the *trans*-1,2-DCE was degraded after 200 hours.[79] In field studies, Semprini et al. found that *trans*-DCE was degraded to a greater degree than either *cis*-1,2-DCE or TCE but was not degraded as fast as vinyl chloride.[80] Laboratory studies by Grbic-Galic et al. (in this volume) indicate that formate may increase TCE degradation in aquifer sediments by providing an alterative source of reducing power that is not a competitive inhibitor of TCE degradation.[81]

Mono- and dioxygenases from other types of bacteria have also been implicated in oxidation of organic contaminants. The ammonia monooxygenase (AMO) enzyme from nitrifying bacteria, *Nitrosomonas europaea,* has also been shown to have the ability to degrade numerous contaminants including aliphatic, cyclic, and halogenated hydrocarbons.[82–84] AMO is structurally similar to MMO,[85] and the mechanisms of degradation may be similar too because the AMO is capable of oxidizing methane and most other MMO substrates. Similarly, the toluene dioxygenase from *Pseudomonas putida* is able to oxidize TCE and related compounds.[86–89]

Aerobic Degradation

Perhaps the best examples of aerobic mechanisms for the degradation of recalcitrant compounds is the current work on PCBs and PAHs. Degradation of the PCBs with few chlorines was described in the 1970s and early 1980s.[90–93] Since then the range of congeners that have been demonstrated to be degraded has expanded and now includes congeners up to hexachlorobiphenyls.[94,95] In a hazard assessment of PCBs, Hooper et al. describe the many problems remaining in the understanding of PCB degradation.[96]

In addition to their role in cometabolic degradation of numerous substrates, mono- and dioxygenase enzymes have also been implicated in the aerobic degradation of PAH by Cerniglia (in this volume).[97] A *Mycobacterium* species capable of growth on phenanthrene has been isolated from estuarine sediment.[98] PAH degradation has been examined in marine sediments, and the rates of degradation have been related to preexposure to related compounds.[99,100]

The importance of acclimation of bacterial to contaminants in both aerobic and anaerobic environments is receiving increasing attention.[101–103] PAH oxidation in marine sediments has been shown to be positively related to oxygen content and to acclimation to high concentrations of PAH for 1 to 2 weeks.[102] In anaerobic lake sediments, the existence of a lag period and subsequent acclimation for degradation of halobenzoates has been noted[104] and characterized.[103] The acclimation effect has also been noted for other compounds in water samples.[105] Schermerhorn and Ventullo (in this volume) have devised a method for examination of acclimation of periphyton communities to organic chemicals.[106] They found that exposure to linear alkylbenzene sulfonate decreased lag times and increased rate constants for degradation.

Genetic Engineering and Molecular Techniques

Potential applications of genetic engineering to degradation of sediment-associated contaminants include changing the regulatory control of degradative genes and providing tools for examination of degradation. The regulatory control of degradative enzymes can be changed to enable production of degradative enzymes without the presence of an inducer (which may compete with the contaminant for sites on the enzyme) or to shift the expression of the enzyme to a nongrowth stage of the bacterial cell growth cycle. Genetic engineering can be also used to provide tools for the examination of the degradative process through the use of "reporter" constructs.

One example of using genetic engineering to alter regulatory control of biodegradative enzymes comes from work with the control of oxygenase expression for degradation of TCE. Typically, degradation of contaminants at low concentrations is difficult due a variety of factors, including, in the case of oxygenase enzymes, competitive inhibition by the enzyme's preferred substrate, which needs to be present to promote production of the enzyme. A change in regulatory control of the enzyme expression can reduce this problem. The toluene-degrading (C1C2BADE) genes from *Pseudomonas putida* were introduced into *E. coli* JM109.[107] The construct degraded TCE at a constant rate, whereas *Pseudomonas putida* degraded TCE at a faster rate initially but at a slower rate at the end of the experiment. Winter et al. cloned the toluene monooxygenase genes into *E. coli* and were able to degrade TCE from 16 ppm to 2 ppb, a level that it is difficult to achieve with nonengineered bacteria.[108]

The use of "reporter" constructs and other molecular techniques may enable researchers to better understand the function of degradative bacteria in sediments. Detection of catabolic genotypes in sediments can give indications of the capacity of the microbial community for degradation of organic contaminants. Tracing of microbial populations capable of chlorinated biphenyl degradation has been demonstrated both in aquatic systems and in terrestrial sediments,[109–111] and the use of DNA probes for detection of metal resistance in soils and aquatic environments has been demonstrated.[112,113] Molecular probes hybridizing with 16S RNA have been developed for the detection of methylotrophic organisms.[114] Sayler et al. (in this volume) describe the use of bioluminescence technology for measuring activity of degradative genes in sediments and also review the information on the occurrence of specific degradative genes in subsurface communities.[115] As pointed out by Knight and Colwell,[43] molecular technology is a rapidly evolving frontier that offers great promise in remediation of environmental contamination.

SUMMARY

This volume will examine key concepts and processes related to biological processes affecting organic contaminants in sediments. We begin with integrative overviews of the physicochemical, biological, and toxicological processes that need to be understood in order to evaluate the fate and effects of hazardous chemicals in sediment-water systems, as well as an overview of new molecular techniques offering great promise for enhancing our capabilities to remediate environmental contamination. Innovative approaches to assessing higher-level ecological responses to contaminants are being developed, and modeling of sediment-water interactions provide valuable insights into the distribution of contaminants on a macroscopic scale; however, it is clear that we have an incomplete understanding of key biological processes controlling the dose of contaminant to which organisms are exposed. The concepts and processes associated with uptake and accumulation of sediment-associated contaminants are divided into two sections focusing on aspects of bioavailability of contaminants, and observations of bioaccumulation of contaminants in natural sediments.

Part IV of this volume is devoted to discussion of biological processes that offer the promise of ameliorating existing problems through biological degradation of sediment-associated contaminants. Chapters are divided into sections focusing on processes of anaerobic dechlorinations and cometabolism of contaminants. The final chapter illustrates an application of molecular techniques in bacteria to better understand the functioning of degradative bacteria in sediments.

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PART II

INTEGRATING CHEMISTRY AND TOXICOLOGY OF SEDIMENT-WATER INTERACTIONS

CHAPTER 2

Making the Transition from Toxicology to Ecotoxicology

James R. Pratt

INTRODUCTION

The sciences of toxicology and ecology are at a crossroads. Toxicological methods have served as quick, convenient, and pragmatic ways of estimating the effects of chemical stressors and mixtures on aquatic life. Ecological risk assessment as practiced by regulatory agencies is, however, nothing more than a series of increasingly sensitive toxicity tests linked together in a tiered or sequential fashion.[1] While these tests examine effects on species of different ecological positions, different life histories, and different longevities, present risk assessment practices in no way evaluate any ecologically meaningful interactions.

Field ecologists have played a major role in assessing environmental damage through biological surveys of the presence, absence, and abundance of a variety of taxa ranging from microorganisms to fish. Concurrent with the "toxicological era" of environmental regulation, a great deal has been learned about the important structures and processes in aquatic ecosystems. The flow of energy through stream ecosystems has been estimated, and the functional role of a variety of taxa within the ecosystems that normally receive our wastes is increasingly better understood.[2] Additionally, our concept of ecosystems has changed to include the importance of energy and nutrient flow and cycling, so that the traditional focus on indicator species[3] holds very little relevance for most ecosystem ecologists.

My purpose is not to review toxicology and applied ecology. Rather, my purpose is to provide a pedagogical review of environmental toxicology as practiced and applied in the regulatory framework, to compare toxicological responses with ecological responses to toxic chemicals, to examine the applicability of ecotoxicological testing, and to make recommendations for adapting and adopting ecological toxicity testing.

A number of professionals like to be known as ecotoxicologists, but the number of practicing ecotoxicologists is small. In fact, it may be a set with no members. An ecotoxicologist applies ecological principles, measures, and information in the evaluation of chemical hazards and risks.[4] I define *hazard* according to a dose-response relationship in which the relative hazard of toxicants is ranked by the dose required to produce a significant, adverse response. A *risk* is defined by hazard and by the probability of exposure of organisms to hazardous concentrations. In protecting aquatic life, ecotoxicological information is not being used. Because of the reliance on sensitive, surrogate species, some chemicals are overregulated with respect to their ecological hazards and risks and some chemicals are underregulated; that is, the best scientific information is not being used to regulate chemicals. This brief chapter in no way summarizes the vast toxicological literature, or even the debate on the appropriateness of tests for regulation. The interested reader is referred to texts that can put these fields in some scientific perspective.[5-7]

TOXICITY AND BIOASSAYS

Toxicity is usually defined as the inherent property of a chemical to produce adverse biological effects. These effects usually are conceived of ranging from acute responses (occurring over a small part of an organism's life cycle), such as mortality or behavioral responses, to chronic effects (occurring over a long period of an organism's life cycle), such as impaired reproduction or reduced growth. The definition of toxicity is critical in the regulatory framework since the general intent, based on the Clean Water Act, has been to eliminate toxic chemicals in toxic amounts from our surface waters. With respect to aquatic life, a chemical is not of concern if it does not appear in the environment or if it appears in such a low concentration that its biological effects are not measurable. Clearly, the definition of toxicity and the means by which it is measured are crucial in environmental protection. If toxicity were defined only as acute toxicity, then allowed environmental concentrations of chemicals would be much higher than if toxicity is defined over a chronic exposure. The ratio of the concentration of chemical that produces acute toxicity to the concentration that produces chronic effects can vary from almost one to several orders of magnitude. A variety of now standard procedures for assessing the toxicity of a chemical has been developed. These procedures—bioassays—use biological material to assess the potential for a chemical to cause damage. The specific mode(s) of action of most toxic chemicals is not known, and so healthy, sensitive organisms must be used to assess biological effects.

Over the years a small group of organisms has been used to assess toxicity (Table 2.1). These organisms serve as surrogates for the larger assemblage of species in an ecosystem, the overwhelming majority of which can never be tested. The surrogates selected are expected to be broadly sensitive, well-studied, and commercially or ecologically important.[7] Ecosystems are quite diverse, and very few species are truly representative of a large number of

Table 2.1. Representative Standard Test Organisms Used to Assess Toxicity

Freshwater	Marine
Fish	Fish
Fathead minnow	Sheepshead minnow
Bluegill sunfish	Mummichog
Rainbow trout	Silverside
Invertebrates	Invertebrates
Daphnids (water fleas)	Grass shrimp
	Mysid shrimp
Algae	Algae
Selenastrum	*Skeletonema*

ecosystems. Perhaps more important criteria in the selection of test species beyond their sensitivity are their availability and ease of culture. This presents a fundamental conflict in toxicology. Test organisms that are sensitive may be fastidious and, therefore, difficult to rear in sufficient numbers for routine testing. The selected group of surrogates is really quite small when one considers that there are probably between 5 and 50 million species on earth and that several thousand of these occur in any ecosystem. Further, the selection of surrogates strongly favors familiar organisms such as fish over smaller, less familiar, but extremely abundant invertebrates and algae.

Two approaches to using bioassays might be envisioned. One would be to test indigenous or representative species from a particular ecosystem that might receive toxic chemicals. Such an approach could lead to regionally specific limits on the exposure of indigenous species to particular chemicals. A second approach, the one that is widely used in regulation today, is to use a fixed set of biological sensors (the standard test species) to test every chemical or chemical mixture. In this way, the biological material is a constant and the relative hazards of individual stressors can be evaluated. Since the procedures are standardized, there is a reasonable assumption of comparability among laboratories, although interlaboratory comparisons have been equivocal.

Deriving water quality criteria or standards is based on the assumption that the sensitivities of species are normally distributed (Figure 2.1) and that by testing species at the sensitive end of the distribution a relationship between dose and the cumulative effects on an array of species can be used to extrapolate to the larger assemblage of species found in ecosystems. While the assumptions might be acceptable if one examines the sensitivities of individual species, it lacks the ability to predict effects on ecological interactions. Further, many compounds must be regulated in the absence of sufficient information. For example, prior to 1980, water quality standards for chronic zinc discharges were based on the toxicity of zinc to rainbow trout because there were insufficient data to relate chronic responses and dose and because rainbow trout are recreationally (and therefore economically) important.[8] Because of the egalitarian nature of regulation, zinc standards were the same all over the country, meaning that even ecosystems that lacked rainbow trout were

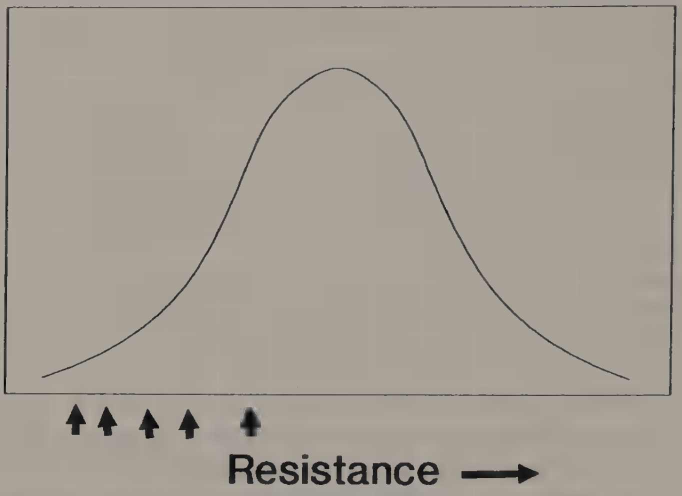

Figure 2.1. Hypothetical distribution of sensitivities to a toxicant. *Arrows* denote the assumed relative sensitivities of common toxicity test species.

regulated at the same zinc concentrations as those that did contain rainbow trout. We now understand that zinc toxicity is affected by water hardness, and zinc criteria differ among soft and hard water streams.[9]

Bioassay information has been used in a standard fashion to develop water quality criteria for over 100 pollutants; however, 6500 chemicals are in common use and may commonly appear in the environment. The bioassay model has been extended to complex mixtures as a way of regulating effluent streams,[10] but because the chemical constituents of an effluent may vary or may not be known, the "dose" can only be expressed in terms of the relative dilution of the effluent by clean water. Nevertheless, the bioassay can differentiate between the relative toxicities evidenced by different compounds or mixtures. Bioassays do not, however, provide direct predictions of ecological effects.

Most toxicological evaluations focus on the responses of small laboratory populations of the surrogate species. While the laboratory populations might serve as surrogates of ecological populations, testing usually focuses on a sensitive life stage (typically juveniles) and so lacks much of the population realism that occurs in the field, where populations have variable age structures and growth is limited by the availability of food and other life requisites. If ecosystems are viewed hierarchically,[11] most of the important ecological processes are a result of interacting populations (Figure 2.2). For example, nutrients are cycled by decomposer food chains. Food chains link species together by predator-prey interactions. Species compete for resources, and those with superior competitive abilities under existing conditions typify certain environments. A large number of ecologists consider themselves to be community ecologists,[12] and although communities as interacting units in the biological

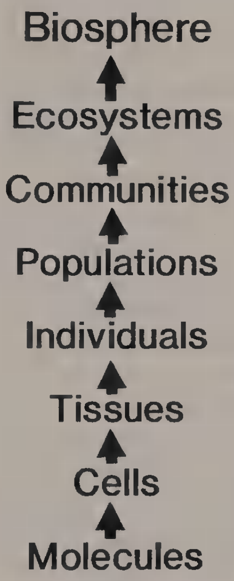

Figure 2.2. The biological hierarchy. Each higher level is assumed to consist of several components from the next lower level.

hierarchy probably do not exist, communities do form understandable subsets of the larger ecosystem.

From a human perspective, ecosystems provide a variety of essential services ranging from biomass production (food and fiber) to waste assimilation. It has never been clearly demonstrated that chemical regulation and the regulation of discharges have resulted in the protection of these ecosystem services. In fact, ecosystems are providing mixed messages to environmental scientists because there are signs of both improvement and continued degradation in related systems. For example, body burdens of DDT and PCBs in lake trout have decreased in Lake Huron[13] but have either remained the same or increased in Lake Ontario over the past decade (Figure 2.3). Commercial fisheries landings show similar confounding responses: catches of some fish have improved, while catches of other fish have decreased significantly (Figure 2.4). It is well known that human influences on terrestrial ecosystems have decreased overall productivity,[14] so it is reasonable to assume that in certain heavily used aquatic ecosystems similar effects might be expected.

Bioassays have been criticized because they lack the ability to predict effects of chemicals at the community and ecosystem level.[5] While the responses of standard test species may vary considerably to a toxic challenge, it would be surprising if the responses of interacting groups of species differed widely from the responses of sensitive surrogate test organisms. In other words, there should be sufficient similarity in the biological machinery so that exposures toxic to standard test species would also be toxic to multispecies assemblages.

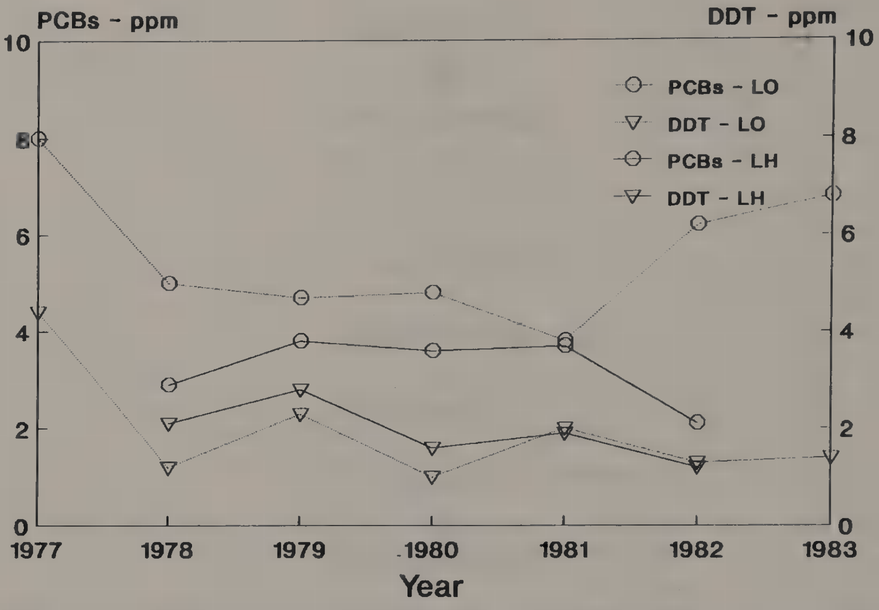

Figure 2.3. Changes in levels of contaminants in lake trout from the Great Lakes. *LO* = Lake Ontario; *LH* = Lake Huron. Adapted from *Environmental Trends*.[13]

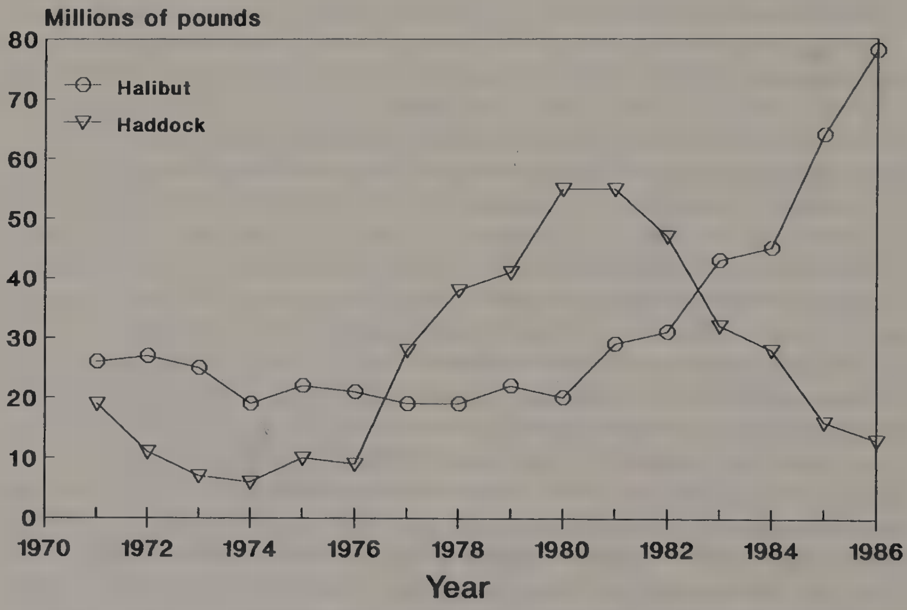

Figure 2.4. Changes in domestic fish landings. Adapted from *Environmental Trends*.[13]

From an evolutionary perspective, metabolic and physiological processes are remarkably constant across all living things. I believe the major point of contention lies not in the prediction of the *concentration* of a particular chemical that might be toxic, but rather prediction of the *magnitude* and *type* of effects that might be observed at the ecosystem level. Since most testing focuses on organisms representative of only a few functions in ecosystems, effects of toxicants on the typically untested species, such as bacteria, other microbes, and plants, are more problematic.

Both toxicologists and ecologists have been somewhat unwilling to accept the concept of laboratory-controlled, ecological test systems where species interactions and ecologically meaningful variables could be measured. Over the past decade it has become clear that laboratory manipulation of species assemblages is not only possible but achievable with sufficiently low variability that important ecological responses can be discerned, both in the investigation of basic questions of ecosystem structure and function and in the applied aspects of anthropogenic influences on ecological phenomena.[15] Ecological test systems should not be expected to be used in the routine way that bioassays are used in chemical regulation.[16] However, laboratory-scale ecosystems can provide important "quality control" checks on predictions from single-species bioassays and can also identify those properties of ecosystems that might be reasonable to measure both in the laboratory and field.

Ecosystems have both collective and emergent properties that result from the occurrence of species in the same place at the same time. *Collective properties* include measures that express the state of an ecological system at a particular time and include population, community, and ecosystem variables. *Emergent properties* are those properties that emerge from the interactions among species and include such processes as predator-prey interactions, nutrient cycling, competition, and succession. These emergent properties are typically measured as rate processes. In a general way, the collective properties of systems can be thought of as measures of the structure of the system, and the emergent properties can be considered measures of system function. In the next section, examples of ecological responses to stress are given and related to conceptual models of expected changes in ecosystems under stress.

ECOLOGICAL TOXICITY TESTING

The diversity of laboratory-scale systems that display ecological properties is quite amazing, ranging from sediment-water systems of only a few milliliters to large channels, tanks, or ponds of around one million liters.[15] Smaller systems have usually been termed microcosms, and larger systems are called mesocosms. The most intensively tested systems are usually a few liters and rarely include organisms as large as fish.[17] Mesocosms that are closer to field scale usually include fish and other large, long-lived organisms.[18] Typically, as the size and longevity of the component organisms increases, experiments must be run over a longer period of time and the cost increases significantly. Artificial pond mesocosms are currently being used to evaluate pesticides.[19]

The criteria for establishing a successful laboratory-scale ecosystem are generally considered to be demonstrations of several ecological properties. Artificial ecosystems should display energy and matter processing, nutrient cycling, and succession; that is, the system should be sustainable and should change with time. For purposes of experimental manipulation and interpretation of results, laboratory-scale systems must be replicable—meaning not that each system is an identical copy of the other, but that variability among systems is sufficiently low that measured properties can be considered similar in the replicates and that during experiments systems develop in approximately the same way.

Approaches to studying the effects of toxic materials on ecosystem structure and function in microcosms have ranged from completely synthetic systems to naturally derived communities. For example, the standard aquatic microcosm (SAM), developed by Taub et al.,[20] assembles an ecosystem from cultured components including protozoa, algae, and crustaceans. The assembled microcosms are dosed with toxic chemicals and are periodically reinoculated from the cultures. This system is nearly totally defined from the culture medium to the component taxa. A second approach, the mixed flask culture (MFC), originally developed by Leffler, assembles a test system from a cultured collection of pond or lake water in a defined medium. Only taxa capable of surviving in the defined medium are eventually apportioned to the microcosms for testing. In the SAM, population dynamics and nutrient pools are measured in response to the toxic dose. In the MFC, functional responses such as primary production and respiration are measured along with nondestructive measures of biomass.

A very different approach, which does not involve culturing, has been used by Giddings et al.,[21] who developed microcosms in large aquaria using sediment, plants, and water from a pond. In this microcosm, the amount of water and sediment are fixed, and a constant wet weight of plants added to each microcosm. No attempt is made to culture or select species; rather, the community is allowed to develop after the microcosm is assembled. By thoroughly mixing sediment and water, there is a good probability of equitable distribution of taxa among replicates. Following the addition of the toxic chemical, microcosms are studied for numbers and kinds of selected taxa, diurnal production and respiration, and major nutrient pools.

Each of the above microcosms uses a static test system. Typically, although not always, the toxic material is supplied in a single dose or a series of pulses. Our laboratory has taken an alternative approach using natural communities and incorporating a continuous input of the diluted toxic material.[22] Microcosms are developed by collecting natural communities and microorganisms on artificial substrata (polyurethane foam) at a reference or unimpacted site in a stream. These communities form the seed material for the replicate microcosms. The toxic chemical is added from a serial dilution device so that the 4-7

L contents of the microcosms are replaced at least five times per day. Communities from the artificial substrata are sampled by removing a substratum and squeezing it into a collecting container. Microcosms are evaluated for species richness of protista, community biomass (protein, chlorophyll) activity of nutrient-transporting enzymes, major nutrient pools, and diurnal production and respiration patterns.

Each microcosm design has unique advantages and disadvantages, and there is not general agreement among ecologists or toxicologists as to the biological significance of certain responses. However, it seems clear that microcosms play an important intermediate role between surrogate species testing and the release of chemicals in ecosystems. Examples of the effects of toxicants on microbial communities in microcosms follow.

Zinc

Zinc is a heavy metal ubiquitous in waste streams. It is known to be bioaccumulated by algae. The water quality criteria for zinc are hardness dependent, but for an intermediate water hardness of 100 mg/L, the chronic zinc criterion for freshwaters is approximately 100 μg/L.[9] Effects of zinc on microbial communities and microcosms showed that species numbers responded quickly and significantly to zinc inputs, resulting in significant depressions of the biota at zinc concentrations above about 90 μg/L (Figure 2.5). Concurrent with the loss of species, both protein and chlorophyll biomass decreased with zinc dose, with chlorophyll showing extremely high sensitivity to the zinc input (Figure 2.5). In other words, algal biomass was severely depressed by zinc addition over ambient. The rate of alkaline phosphatase activity, a measure of the ability of the microbiota to recover phosphorous from organic compounds, was enhanced as phosphate pools in the test systems dropped (Figure 2.6).

It is worth noting that most of the significant responses in the test systems occurred at zinc levels below current water quality criteria and that biomass responses were significant at levels of more than one order of magnitude lower than the current standard (Table 2.2). Summaries of previous research have shown that microcosm responses are comparatively sensitive when compared to the responses of surrogate species.

Atrazine

Atrazine is not a priority pollutant whose environmental levels are regulated as are many compounds discharged from point sources. Atrazine enters the environment primarily from agricultural and horticulture uses because it is a commonly used herbicide. Atrazine comprises a significant proportion (greater than 10%) of the annual poundage of pesticide used in the United States. It is widely used as a preemergence herbicide. Related triazine herbicides find diverse uses, from clearing rights of way to aquatic plant control.

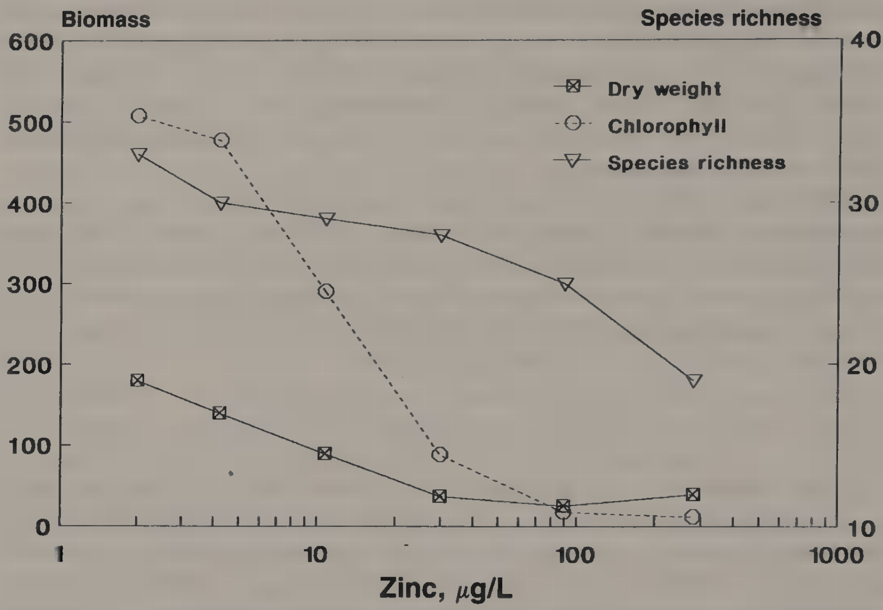

Figure 2.5. Effect of added zinc on community structure of laboratory microcosms. Species richness data are for protozoa. Dry weight units are µg/mL. Chlorophyll was measured as fluorometric units. Controls had 2 µg Zn/L but are plotted as 2 µg/L. Points are means of triplicates.

All of these herbicides are photosynthetic inhibitors affecting the Hill reaction and electron flow of photosystem II.

Our investigations of atrazine toxicity in microcosms showed unexpected patterns, including the stimulation of biomass production and increases in species number (Figure 2.7). At elevated doses of atrazine, alkaline phosphatase activity increased dramatically as phosphorus was lost from experimental systems (Figure 2.8). Although biomass was stimulated, the production of oxygen in experimental systems did not increase, suggesting that although more chlorophyll was present, no additional primary production was taking place. Presumably, the elaboration of chlorophyll biomass was a response to the inhibition of photosynthesis by the added atrazine. Increases in species richness can only be interpreted as indicative of the breakdown of normal control mechanisms in communities. Interestingly, these effects of atrazine occur at concentrations that have been measured in the field in areas where atrazine is widely used as an agricultural chemical.

These examples are not intended to show the superiority of a particular testing system, but demonstrate the potential for ecologically meaningful measures of complex interacting communities to be made under laboratory conditions using experimental designs similar to those used in surrogate species testing. Modeling the dose response of toxic chemicals is critical to conducting

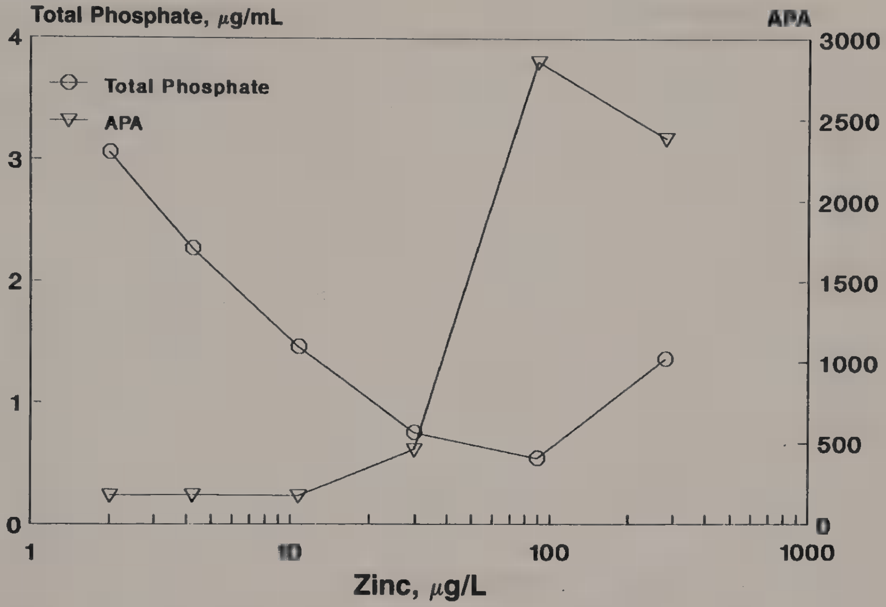

Figure 2.6. Effect of added zinc on nutrient cycling in laboratory microcosms. Units for alkaline phosphatase activity (APA) are nmoles *p*-nitrophenol/mg protein/hr. Controls had $<2\mu g$ Zn/L but are plotted as $2\mu g/L$.

quantitative risk assessments, and it is now clear that ecological measures can be used to differentiate adverse responses from normal variability in communities. Ecological responses are not as variable as had once been anticipated, assuming that adequate experimental design, sampling, and analysis are exercised to improve detection power.

Table 2.2. Summary of Zinc Toxicity in Naturally Derived Microcosms. [Table values are μg Zn/L. The chronic value (ChV) is the geometric mean of the lowest observable effect concentration (LOEC) and the no observable effect concentration (NOEC) determined by comparisons to controls. The EPA water quality criterion is shown for water hardness of 65 mg $CaCO_3$/L, the water hardness of the experimental systems. The NOEC could not be calculated when all responses differed from controls.]

Variable	NOEC	LOEC	ChV
Species richness	89.2	280	158
Dryweight	—	4.2	—
Chlorophyll *a*	4.2	10.7	6.7
Dissolved oxygen	—	4.2	—
Alkaline phosphatase activity	29.8	89.2	51.6
EPA criterion			73.6

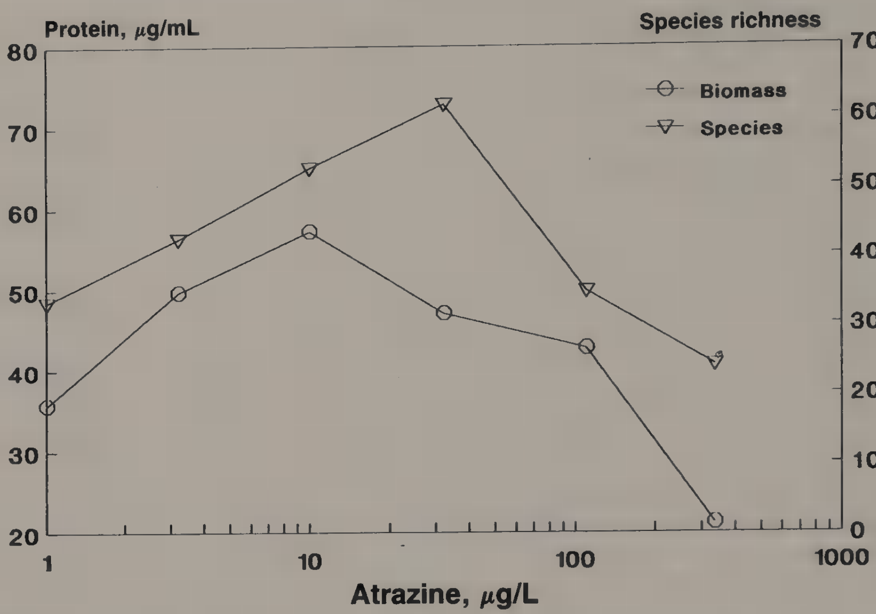

Figure 2.7. Effect of atrazine on community structure of laboratory microcosms. Species richness data are for protozoa. Biomass data are total protein (µg/mL). Controls are plotted as 1 µg atrazine/L.

EXPECTED CHANGES IN STRESSED ECOSYSTEMS

If microcosms are valid test systems, their responses should be congruent with predictions and observations of ecosystem response to stress. This congruence does not validate the microcosm approach to evaluating ecological toxicity, but does show that microcosms can respond in a manner that might be anticipated by observers of larger systems. Several recent papers have reflected on the types of changes that might be anticipated in stressed systems, although not all of these anticipated changes are readily observed.[23-25]

Species richness

Species are usually considered to be normally distributed (Figure 2.9) in most communities.[26,27] Chemicals affecting communities usually reduce the abundance and diversity of taxa. Occasionally, species numbers are stimulated, and both effects result in a deviation from the nominal state, indicating adverse ecological effects.

Biomass

Toxic influences usually reduce standing crop, but stimulation is not unknown since resistant species or groups of species may be capable of exploiting stressed environments when normal competitive controls are released.

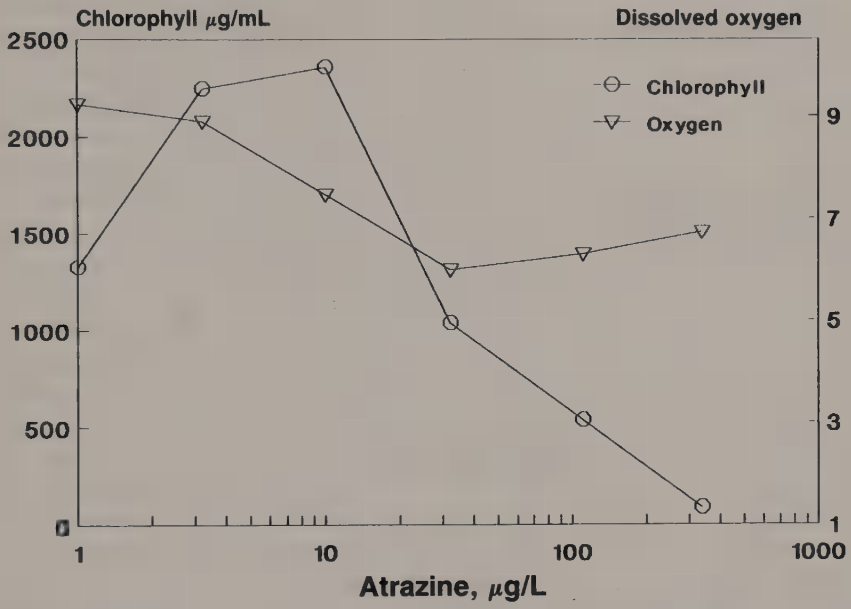

Figure 2.8. Effect of atrazine on chlorophyll biomass and midmorning dissolved oxygen levels in laboratory microcosms. Chlorophyll units are μg chlorophyll *a*/L. Dissolved oxygen units are mg/L. Controls are plotted as 1 μg atrazine/L.

Primary production

The photosynthetic machinery of most ecosystems is sensitive to the abundance of photosynthetic individuals and the availability of nutrients. Like biomass changes, productivity may be depressed by toxic chemicals or enhanced by the removal of competitors. While primary productivity responds to both nutrient enrichment and toxicity, primary production is usually limited by the availability of nutrients. In aquatic systems affected by toxicants, primary productivity may respond more to nutrient limitation than to toxicity. For example, in a stream that had 50 algal taxa whose primary productivity was limited by phosphate availability, the effect of a toxicant that removed all but 10 taxa but had no effect on the supply of phosphate might be observed to have no effect on production.

Productivity measures are further complicated by methodological limitations, so that only relatively gross changes can be detected. When biomass is low, radiotracer techniques may require relatively long incubation times to achieve detectable uptake of labeled compounds. The resulting measure of primary production often has a coefficient of variation as high as 100%. We have attempted to measure diurnal production and respiration patterns indirectly in microcosms using continuous measures of pH and have discovered

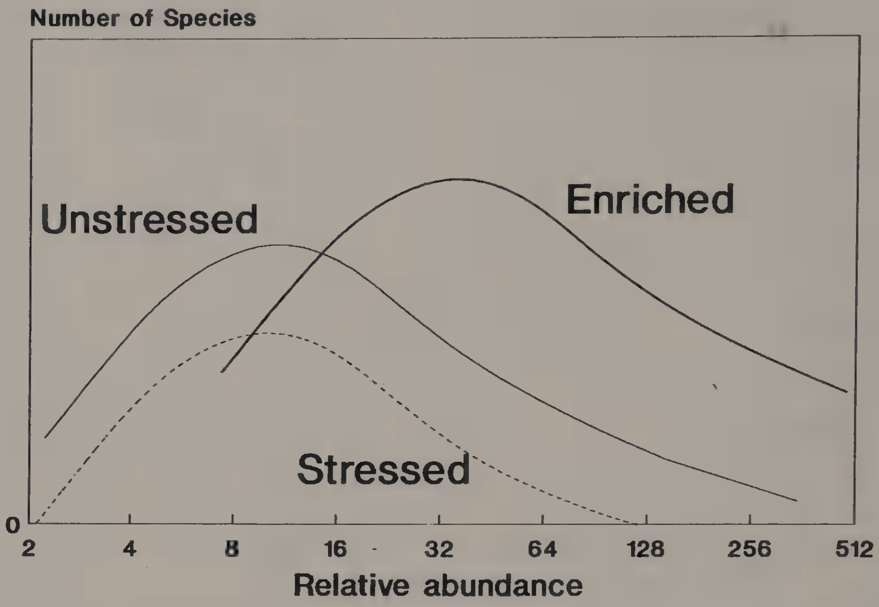

Figure 2.9. Changes in relative abundance and numbers of species in communities under stress. Enriched communities often show large population sizes of insensitive species. Stressed communities show depressed population sizes and species richness when compared to unstressed reference communities. From Preston[26] and Patrick.[27]

that not only are production and respiration affected by toxic action, but the coupling of processes clearly decreases as toxicity increases.

Energy flow

As primary production is inhibited in ecosystems, there are increasing trends toward heterotrophy. In most stable ecosystems there is a balance between production and respiration. However, as toxicity depresses primary production, decomposer pathways can dominate.

Macronutrients

Ecosystems under stress commonly are unable to recover nutrients that are usually tightly cycled and the systems become "leaky" (e.g., Figure 2.6). Similarly, as systems shift from plant dominated (where potassium levels are high) to animal dominated or decomposer dominated, system pools of a variety of nutrients can shift.

Homeostasis

At the extremes of stress effects, systems are no longer able to compensate for small perturbations. The inability of systems to maintain structure and function under stress is simply the cumulative effect of all the responses listed above: changing species richness, changing standing crop, and changing productivity dynamics.

Unfortunately, ecosystem stress is widespread, and ecological studies have not characterized a sufficient diversity of ecosystems for us to understand the normal operating range of most ecosystems. Additionally, it must be remembered that the development of every ecosystem is a unique, historical process, and although ecosystems share many common structures and functions, the diversity of even neighboring ecosystems is a result of numerous processes that are not understood. Regional approaches to understanding the normal structures in terms of species composition of ecosystems may provide some baseline against which to compare future changes or current conditions, but it is unlikely that we will have good information on the range of processes and the normal community structure at a variety of taxonomic and trophic levels for very many ecosystems unless there is a significant change in the research interests of funding organizations.

To a cynical mind, this ignorance is blissful because we have considerable difficulty detecting when ecosystem changes have occurred. Evidence now clearly points to the importance of understanding the community and population dynamics of small, rapidly reproducing, poorly dispersing species as indicators of ecosystem change. Interestingly, the biology and ecology of these species has been wantonly ignored by all but a small number of professionals, so that the expertise is not available to assess many ecosystems. Further, taxonomic skill is rapidly disappearing in the biological sciences, so that our ability to monitor ecosystems by the species present is compromised. Even if we were to suddenly return to the biological survey as a way of evaluating ecosystem conditions, it would be difficult to identify many taxa since work on their systematic position, identity, tolerances, and distribution essentially stopped 20 years ago.

SUMMARY AND CONCLUSIONS

The single-species bioassay remains the workhorse of environmental toxicology. Surrogate species are used to rank the relative hazards of chemicals in both acute and chronic exposures. Such standard assays, when coupled with information about the environmental fate of chemicals, are a useful first step for establishing standards for allowable environmental concentrations of potentially toxic materials. However, ecological information is increasingly needed both in sensing the condition of the environment and in checking the accuracy of predictions based on surrogate species testing. Surrogate species cannot be used to predict the kinds of ecological changes that might occur from the exposure of complex ecosystems to toxic materials, even though there is some consistency in the innate biological machinery of all living organisms. Some toxic materials may be bioaccumulated and bioconcentrated and produce harmful effects that cannot be predicted from short-term laboratory tests. Other chemicals may be relatively harmless in the environment because they may be rapidly metabolized or in other ways sequestered in the environment. Ecological toxicity testing can serve as an intermediate step between surrogate species testing and environmental release. Laboratory-scale ecosystems are used to evaluate ecological responses in ecosystem-like settings. For conservative chemicals, such as heavy metals or organic materials with long environmental persistence, ecological testing can serve as a necessary quality control step in standard setting. For nonconservative pollutants, ecological testing can reveal the degree to which toxicity can be ameliorated by ecosystem processes such that presumptions of hazard may be reduced.

At the present time, ecological testing is sufficiently well developed to be used in site-specific cases or in cases where additional, ecologically meaningful information is needed to evaluate environmental effects of chemicals. However, considerably more study is needed to understand the relative vulnerability of differing ecosystems so that standards may vary regionally based on ecological characteristics rather than simple measures such as water hardness. The factors that influence ecosystem variability are not well understood, but ecological toxicity testing using natural communities could certainly unravel these problems. Additional work is needed to understand the natural variability of ecosystems so that ecologists and environmental scientists can distinguish between anthropogenic changes and natural oscillations in the ecosystems. Despite the ability to measure a variety of biochemical changes in systems, the identities of component species continue to be important, and failure to adequately characterize the taxonomic composition of ecosystems will result in poor detection power for scientists endeavoring to protect ecosystem structure and function.

ACKNOWLEDGEMENTS

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CHAPTER 3

The Case for Modeling Sediment-Water Interactions in Aquatic and Marine Systems

Donald Mackay, Miriam Diamond, and Warren Stiver

INTRODUCTION

There are numerous situations in which aquatic and marine sediments have become contaminated with metals and organic chemicals as a result of high levels of past chemical discharge. When discharges are reduced or eliminated, bottom sediments may cease to act as a net sink for contaminants and become in-place sources of contamination. Ecosystem recovery may then be retarded as chemical "bleeds" steadily from the sediments to the water column. In this chapter, we argue that when assessing the present condition of such systems, and especially when deciding on remedial measures, it is important to understand, as quantitatively as is possible, the dynamics of the contaminants in the system. This includes information on where the contaminant resides, which processes are responsible for transformation and transport between water, sediment, and biota living in both media, and, thus, how long it may take for the system to become restored naturally, in response to remedial interventions. Much of this information can, and usually is, obtained by monitoring programs, in which sediments, water, and biota are sampled and analyzed and the analytical results interpreted, but there are compelling arguments for proceeding to an even higher level of sophistication of data interpretation by compiling a mass balance model in which the rates of chemical transport and transformation are estimated. If a reliable model is available, it becomes possible to test various remedial strategies, and essentially play "environmental video games" on the computer. We believe that this procedure helps to obtain and transmit a higher level of understanding of the nature of the system and how it may respond to natural processes and to remedial measures. A comprehensive recent treatment of this issue can be found in the compilation of studies by Hites and Eisenreich.[1]

In essence, monitoring efforts yield information about concentrations that are a snapshot in time of a set of complex, dynamic, interacting processes. By assembling a model, we attempt to create a quantitative picture of the often unmeasurable and invisible dynamic processes of transport and transformation that result in these concentrations and ultimately cause ecologically adverse effects.

Our aim in this chapter is first to present a simple sediment-water interaction model, discuss various aspects and applications of the model, and explore some of its implications for probing the nature of sediment-water systems. We first assemble a relatively simple linear mass balance model in which chemical is exchanged between a well-mixed sediment layer, a well-mixed overlying water layer, and the atmosphere. We then apply the "linear additivity principle" of Stiver and Mackay[2] to this model as a method of discriminating between sources of contaminant in various components of the ecosystem, and especially the contribution of in-place pollution. We suggest that the primary incentive for efforts of this type is to gain an improved appreciation of the system for management and remediation purposes. The analysis highlights the importance of developing an improved capability of modeling sediment-water exchange processes, because it is these processes that usually control the remediation or recovery time of sediments. Accordingly, in the second part we examine in some detail a more narrowly focused model of sediment-water exchange and address questions such as:

- Which chemicals are of concern as potential in-place contaminants?
- What is the role of biomonitoring?
- Which are the most important transport and transformation processes?
- How can we best probe sediment-water dynamics and estimate process rates?

To accomplish this task, we use a simple evaluative model, similar in dimensions and properties to Lake Ontario. This enables us to draw on several recent studies of this system, notably that of Mackay.[3,4] We first examine the fate of a chemical similar to PCBs in the system and later examine the effect of changing chemical properties. Although an approximation to Lake Ontario is used as an example, it is believed that the principles presented here are applicable to other lakes, and even to near-shore and estuarine marine systems.

MODEL STRUCTURE

When assembling models of this type, we prefer to use the fugacity or aquivalence formalism as described in a series of papers by Mackay et al.,[5] Mackay and Paterson,[6] Mackay and Diamond,[7] Reuber et al.,[8] Diamond et al.,[9] and Mackay.[3,4] The fugacity approach has been described in these studies, and only a brief review of salient features is presented here. It should be emphasized that the same results can be obtained using conventional concentration-based models.

The partitioning of chemical between various phases is characterized by Z values, one Z value quantifying the capacity of each medium for each chemi-

Table 3.1. System Dimensions, Chemical Properties, Z and D Values for PCBs

System Dimensions	
Lake area (m^2)	1.95×10^{10}
Water depth (m)	86
Sediment depth (m)	0.005
Water particles (g/m^3)	0.5
Sediment porosity	0.85
Particle OC content	0.2
Sediment OC content	0.0359
Solids density (kg/m^3)	2400
Chemical Properties	
Molecular mass (g/mol)	326
Henry's law constant (Pa m^3/mol)	12.2
Log K_{ow}	6.6
Z Values (mol/m^3 Pa)	
Z for air	0.0042
Z for water	0.0818
Z for water particles	64100
Z for sediment particles	11500
Z for biota	18900
D Values (mol/Pa hr × 10^{-6})	
Burial (DB)	2.29
Sediment reaction (DS)	0.34
Sediment resuspension (DR)	2.26
Water-sediment diffusion (DT)	0.64
Sediment deposition (DD)	30.5
Water transformation (DW)	0.79
Air-water diffusion (DV)	5.43
Water inflow (DI)	1.98
Water particle inflow (DX)	15.2
Water outflow (DJ)	1.98
Water particle outflow (DY)	0.38
Rain dissolution (DM)	0.14
Wet particle deposition (DC)	14.7
Dry particle deposition (DQ)	6.1

cal. Z values, with dimensions of mol/Pa m^3, are proportionality constants that relate concentration (C, mol/m^3) to fugacity (f, Pa), i.e., $C = Zf$. They are deduced from the physico-chemical properties (especially the partition coefficients) of the chemical. The phases or media treated here are air, aerosols, water, suspended particulate matter, sediments and pore water, and biota in the water column and in the sediments. Z values can also be defined for bulk or mixed phases such as water containing suspended matter. The properties and Z values of a PCB-like chemical illustrated here are listed in Table 3.1, the values being essentially those suggested by Mackay.[3]

Transport and transformation rates (with units of mol/hr) are characterized by D values (with dimensions of mol/Pa hr); for example, the rate of each process is Df, the product of a D value and a fugacity. D values are calculated from quantities such as flow rates, mass transfer coefficients, and diffusivities as described earlier by Mackay.[3] The total quantity of chemical in a well-mixed medium is then VC (mol), where V is volume (m^3). This amount is also VZf (mol).

In the interest of simplicity, it is assumed that air is in equilibrium with aerosol particles. water is in equilibrium with water column particles and biota, and the bottom sediment solids are in equilibrium with pore water and sediment biota. There are, thus, three fugacities in the system that characterize the concentrations throughout the system. A listing of the system dimensions, Z values, and D values, including definitions, is given in Table 3.1.

Mass balance differential equations can be written for the sediment and water compartments by equating the rate of inventory change of chemical in the system to the sum of the input rates less the sum of the output rates, resulting in Equations 3.1 and 3.2 below. The rate of direct discharge of chemical to the water is E (mol/hr), and the subscripts of fugacity f (Pa) refer to water W, inflowing water I, sediment S, air A, total water phase (including particles) WT, and total sediment phase of solids and pore water ST.

$$V_W Z_{WT} df_W/dt = f_I(DI + DX) + E + f_A(DV + DM + DC + DQ) + f_S(DR + DT) - f_W(DJ + DY + DV + DW + DD + DT) \quad (3.1)$$

$$V_S Z_{ST} df_S/dt = f_W(DD + DT) - f_S(DR + DT + DS + DB) \quad (3.2)$$

Also of interest are the steady-state versions of these equations, which can be obtained by setting the derivatives equal to zero. This yields Equations 3.3 and 3.4 below, which can be solved to give Equation 3.5, in which the water fugacity, f_W, is a function of only the input parameters. The sediment fugacity, f_S, is calculated from Equation 3.4.

$$f_W = \frac{f_I(DI + DX) + E + f_A(DV + DM + DC + DQ) + f_S(DR + DT)}{DJ + DY + DV + DW + DD + DT} \quad (3.3)$$

$$f_S = \frac{f_W(DD + DT)}{(DR + DT + DS + DB)} \quad (3.4)$$

$$f_W = \frac{f_I(DI + DX) + E + f_A(DV + DM + DC + DQ)}{DJ + DY + DV + DW + \dfrac{(DD + DT)(DS + DB)}{(DR + DT + DS + DB)}} \quad (3.5)$$

$$f_W = \frac{f_I(DI + DX)}{DTOT} + \frac{E}{DTOT} + \frac{f_A(DV + DM + DC + DQ)}{DTOT} \quad (3.6)$$

where DTOT is the sum of the D terms in the denominator of Equation 3.5.

A noteworthy feature of these equations is the presence in the numerator of Equation 3.5 of the three input terms to the system as a simple linear addition, i.e., the inputs from water inflow to the system, direct discharges, and from the atmosphere. Equation 3.5 can thus be rewritten in the form of Equation

3.6 as the sum of three separate terms, each representing that part of the water fugacity attributable to the specific input term. Since the sediment fugacity and the sediment biota fugacities and concentrations are directly related to the water fugacity, it is clear that the concentrations in these compartments and the chemical masses attributable to the specific inputs can also be calculated individually and added to give the total. The model thus permits the total fugacities (and hence the concentrations) to be decoupled into contributions from each source, i.e., "blame" can be assigned quantitatively.

For illustrative purposes, concentrations can also be calculated in water column and sediment biota by assuming equi-fugacity and a lipid content of 5%, i.e., the bioconcentration factor is 0.05 K_{ow}, where K_{ow} is the octanol-water partition coefficient. This attribution of concentrations to sources is only possible if all expressions in the model equations are linear.

APPLICATIONS OF THE WHOLE LAKE MODEL

Figure 3.1 and Table 3.2 give the results of the steady-state mass balance calculation (Equations 3.4 and 3.5) for assumed chemical inputs in air (as f_A or C_A), in water (as f_I or C_I), and by direct discharge (E). The important processes are clearly deposition, resuspension, volatilization, and burial. Quite different fate profiles would be obtained for other chemicals that are more volatile, or reactive, or less hydrophobic. Figure 3.1 contains a wealth of process information not available from perusal of concentration data alone.

Table 3.2 consists of rows corresponding to selected output quantities from the model, such as concentrations, masses, and fugacities. These quantities are regarded as being the key descriptors of the condition of the chemical within the system. The columns represent values obtained as a result of various calculations. The first column is observed or monitored data for the status of the chemical at a particular point in time, typical of data which would be obtained as a result of a conventional monitoring program. The values are similar to those reported for PCBs in Lake Ontario in the mid-1980s.[3] Column 2 and the data in Figure 3.1 are the result of steady-state calculation of the concentrations and fugacities assuming all three inputs to apply, i.e., using Equations 3.3 and 3.4. Column 3 gives the values obtained by assuming only inputs by direct discharge. Column 4 treats only input by advective inflow, and Column 5 has input only from the air. Essentially, columns 2, 3, 4, and 5 are the results of applying Equation 3.6, first in total, then term by term.

It is obvious by inspection of both the numbers and their algebraic origin that columns 3, 4, and 5 add to give column 2. It is thus possible to assert that the calculated steady-state concentration in the ecosystem as calculated in column 2 is attributable 86% to water inflow, 4% to direct discharges, and 10% to atmospheric sources.

Column 6 is obtained by subtracting column 2 from column 1. This set of quantities represents mathematically the unsteady-state contributions to the

Table 3.2. System Response to Various Inputs

	Col. 1 Observed Concentrations	Col. 2 All Inputs	Col. 3 Discharge Only	Col. 4 Water Inflow Only	Col. 5 Air Concentration Only	Col. 6 Unsteady Contribution
Input Data						
Air concentraion (ng/m^3)	—	0.512	0	0	0.512	
Water inflow concentration (ng/L)	—	10	0	10	0	
Discharges (kg/year)	—	100	100	0	0	
Air source (kg/year)	—	254	0	0	254	
Water source (kg/year)	—	2097	0	2097	0	
Water Column Data						
Concentration (ng/L)	1.5	1.14	0.04	0.97	0.12	0.36
Fugacity (nPa)	48	36.7	1.5	31.4	3.8	11
Mass of chemical (kg)	2500	1900	77	1626	197	600
Bottom Sediment Data						
Concentration (ng/g)	500	323	13	276	34	177
Fugacity (nPa)	320	207	8.4	177	21.4	113
Mass of chemical (kg)	17500	11310	461	9675	1174	6200
Biotic Data						
Water col. concentration (μg/g)	0.3	0.19	0.01	0.16	0.02	0.11
Sediment concentration (μg/g)	—	1.10	0.04	0.94	0.11	—
Percent of Col. 2	—	100	4.0	85.6	10.4	—
Percent of Col. 1 (Sediment concentration)	100	65	3	55	7	35

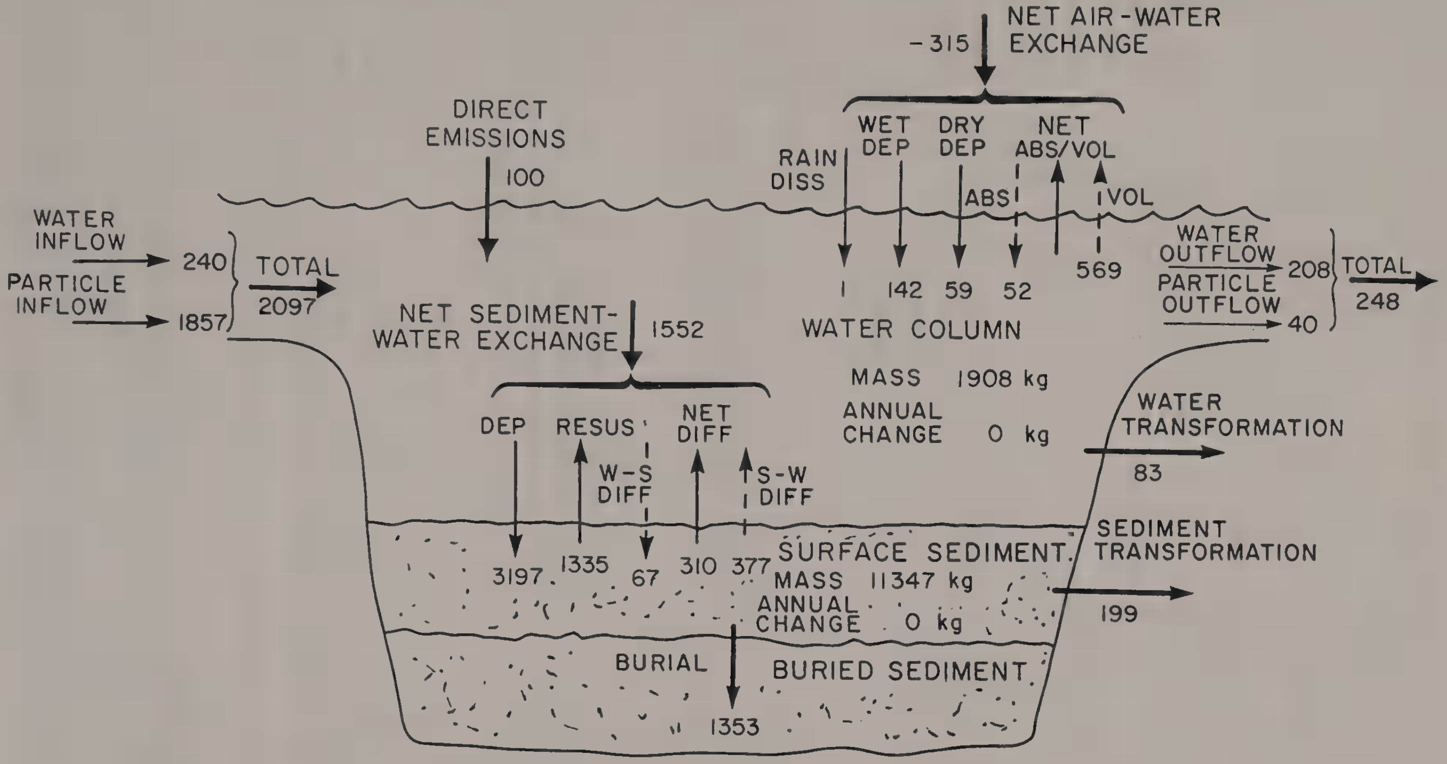

NET AIR-WATER EXCHANGE
-315
DIRECT EMISSIONS
100
RAIN DISS
WET DEP
DRY DEP
NET ABS/VOL
ABS
VOL
WATER INFLOW
240
PARTICLE INFLOW
1857
TOTAL
2097
1
142
59
52
569
WATER OUTFLOW
208
PARTICLE OUTFLOW
40
TOTAL
248
NET SEDIMENT-WATER EXCHANGE
1552
WATER COLUMN
MASS 1908 kg
ANNUAL CHANGE 0 kg
DEP
RESUS
W-S DIFF
NET DIFF
S-W DIFF
WATER TRANSFORMATION
83
SEDIMENT TRANSFORMATION
199
3197
1335
67
310
377
SURFACE SEDIMENT
MASS 11347 kg
ANNUAL CHANGE 0 kg
BURIAL
1353
BURIED SEDIMENT

Figure 3.1. Comprehensive steady-state mass balance with all rates in kg/year.

present condition. It also contains any error that may be present in the model; however, for purposes of this discussion we assume that the model is correct and that the difference is entirely attributable to the unsteady-state nature of the response of the system. The agreement between the water concentrations in columns 1 and 2 is fairly good and could be within experimental error, but there is obviously a severe discrepancy between the sediment concentrations. This is probably attributable to past discharges at higher-than-present levels that caused, at that time, higher water and sediment concentrations. The sediment has retained the memory of these higher discharge rates. For example, in this case it appears that discharges in the recent past may have been about twice the current estimated values, resulting in about twice the present calculated steady-state sediment concentration. Neither the equations nor the quantities in column 6 contain any information about when these discharges took place or their magnitudes. The amounts in column 6 represent an excess amount, now present, over values that would eventually prevail if current levels of input were to be maintained over time. In the event that air inflow and discharge rates were maintained indefinitely at the values used to deduce column 2, the system would adjust eventually to the values in column 2. The contribution in column 6 would gradually decay to zero, as depicted in Figure 3.2.

In practice, because there is error in the analyses and the model, and spatial variability in the aquatic ecosystem, it is more rigorous to present the data in columns 1 to 6 as best estimates with confidence intervals. This will help avoid over-interpreting the data and may show, for example, that certain column 6 quantities may be zero.

Of obvious interest is the time that will be required for decay of the unsteady-state component to take place; that is, will it be 1 year or 20 years? Determination of an accurate response time requires numerical integration. An approximate response time can, however, be deduced by mere inspection of Equations 3.1 and 3.2. These equations take the form of Equation 3.7 below:

$$\mathrm{VZ}\ df/dt = \mathrm{I} - f\,\mathrm{D_E} \tag{3.7}$$

where I is the sum of the input terms and D_E is the sum of the D values by which chemical can leave the compartment; that is, it is the total "exit" D value. Assuming I to be constant and integrating from initial fugacity f_O gives

$$f = \mathrm{I/D_E} + (f_O - \mathrm{I/D_E})\exp(-\mathrm{D_E}\,t/\mathrm{VZ}) \tag{3.8}$$

Clearly the rate constant is D_E/VZ. Its reciprocal, the characteristic time, that is, the time to approach to approximately 70% of equilibrium or steady state, is VZ/D_E. In this case the response times are 0.46 years for water and 3.5 years for sediment. In reality these are minimum response times since they contain the assumption that chemical that has left a compartment, such as sediment, will not return to that compartment. In practice, contaminant released from

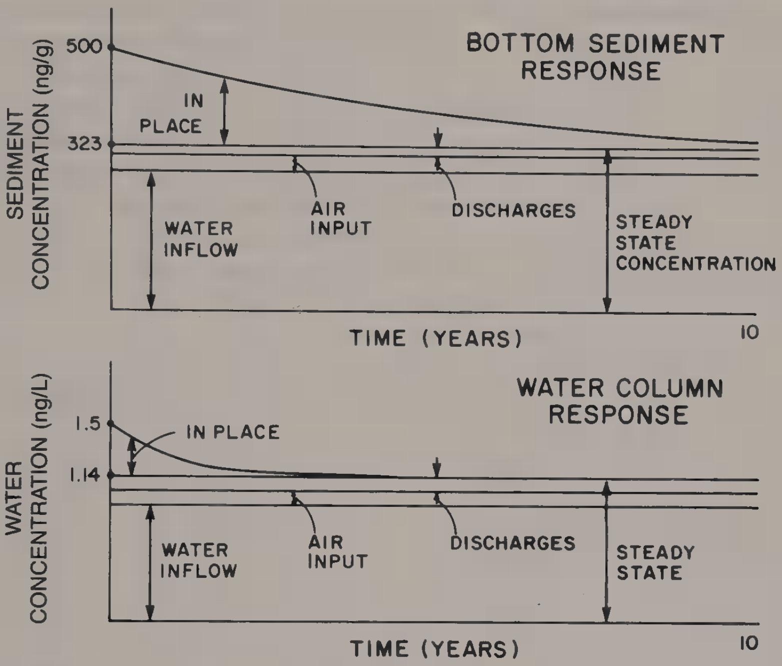


Figure 3.2. Illustrative unsteady-state response of bottom sediment and water column to a new steady state, showing the relative proportions of the in-place water inflow (Niagara River), air inputs, and direct discharges. The more rapid water response is apparent.

the sediment to the water will return, at least to some extent, to the sediment to prolong the recovery time. A more realistic and longer recovery time can be estimated by multiplying the above time by a factor corresponding to the fraction of the material that, on leaving the compartment, will never return. If this is done it increases the sediment response time to 6 years. Obviously, the sediment response time is directly dependent on the sediment volume or depth selected, which in this case is only 5 mm. Increasing this by a factor of ten, to 5 cm, would cause a tenfold increase in response time; that is, clearance would take ten times longer. This depth is clearly a critically important parameter in controlling the recovery time.

The most reliable way of estimating these recovery times is to undertake a numerical integration of Equations 3.1 and 3.2, calculate the changing concentrations over time, and read off the response time from a plot similar to that in Figure 3.2. The value obtained should be similar in magnitude to the response times deduced approximately above. Analytical solution is also possible, but the resulting equations are too complex to be easily interpreted.

It is now possible to assert that the present sediment concentration as observed in column 1 can be broken down as follows: 55% is attributable to advective Niagara River inflow, 3% is attributable to current discharges, 7% is attributable to atmospheric sources at present concentration levels, and 35% is attributable to in-place contamination resulting from previous, higher-than-present loadings. Corresponding values can be obtained for water and biotic concentrations. It is believed that this calculation can be very useful from a management point of view because it indicates the extent to which recent and planned measures to reduce the sources of contamination will be sufficient to achieve (eventually) desired low levels. For example, if in-place contribution is 90% of the total, there is probably little justification for reducing present inputs further. If the in-place contribution is only 20%, then further reduction of inputs is obviously indicated. The model thus assists the regulator in assigning resources in the most effective way, i.e., to source reduction, or to attempts to remediate in-place contamination. There is little merit in remediating if the inputs are such that recontamination will occur.

Obviously of critical importance in this decision is the response time of the sediment, but in some cases it may also be important to assess the water column response time. The simplest method of assessing the relative contributions of the water and the sediment is to calculate the mass of contaminant in each compartment. In this case, the water contains 12% of the contaminant, and the sediment 88%; thus, it is clear that most of the contaminant is bound up in a slowly responding sediment compartment, and it is the rate at which this compartment can clear chemical that will control the system's recovery. It is on the detailed determinants of this rate of clearance or recovery that we focus next.

SEDIMENT-WATER EXCHANGE PROCESSES

In this section, we explore the mechanisms of transport of chemicals of varying hydrophobicity between the water column and the sediment. This analysis is similar to that of Diamond et al. for radio-isotopes with varying sorptive tendencies.[9] We apply the model developed earlier with the same parameters, but we allow the chemical's octanol-water partition coefficient (K_{ow}) to vary from 10^3 to 10^8, all other properties being identical. This has the effect of changing the D values for any process in which the chemical is partitioned, transported, or transformed in an organic phase, notably the bottom and suspended sediment and biotic phases. We assume that there is a constant total concentration in the water column in all cases of 1.14 ng/L of chemical, and we calculate the steady-state sediment fugacity and concentration from this water concentration using Equation 3.4. Biotic concentrations are also deduced by assuming equifugacity with the residing medium.

It is useful at this stage to examine the terms in Equation 3.2 in more detail. Transport from the water column to the sediment occurs by two mechanisms, diffusion in the water column (described by DT) and transport associated with settling organic matter (described by DD). Transport of chemical from water to sediment is thus accomplished in parallel in two distinct phases, in solution in water and in association with particulate organic carbon. The relative importance of these transfer routes depends on the organic carbon to water partition coefficient K_{oc}, which depends on K_{ow}, the octanol-water partition coefficient. When K_{oc} or K_{ow} is large, it is expected that most transfer will take place in association with particulate organic carbon. When K_{ow} is small, the transfer will be primarily in water solution. It is not, however, clear over which ranges of K_{ow} values one or the other mechanism will predominate. We do not treat any partitioning or transport influenced by dissolved or colloidal organic carbon.

Transport of contaminant from sediment to water also occurs by two parallel processes: in solution in water, described by DT, and in association with particulate organic matter, described by DR. The resuspension term DR includes material conveyed from the sediment layer back into the water column as a result of disturbances of a hydrodynamic or biological origin, and it could be modified to include diffusion of organic colloids containing absorbed chemical. DR is thus viewed as expressing the sum of all processes conveying chemical from sediment to water in association with organic matter, whether the organic matter be on sediment solids or in colloids. Again, as K_{ow} increases, it is expected that most chemical transport from sediment to water will be in association with these organic phases.

It is noteworthy that DD is about ten times DR; that is, deposition is potentially ten times faster than resuspension. There are two reasons. The mass of depositing material is greater, because some is buried and thus fails to be resuspended. More importantly, the organic carbon content of the settling material is 20%, compared with only 3.6% for the resuspended, partly mineralized material.[3] Hydrophobic chemicals are thus readily conveyed to the bottom but have difficulty returning to the water because of the lack of available organic carbon carrier. It is assumed that when the organic carbon is mineralized, the chemical is "freed" and partitions onto the remaining organic carbon, or remains in the pore water.

The other two chemical removal processes in the sediment, burial and reaction, apply to the bulk chemical in the sediment regardless of whether it is sorbed to solids or associated with the pore water. Generally, however, because of the relatively high solids concentration in the sediment of typically 10 to 30%, most chemical will be associated with solids and very little will be in dissolved form. This contrasts with the water column, in which generally most chemical will be in dissolved form, and only when K_{ow} is very large, will there be a significant fraction of the chemical associated with the particulate and colloidal phases.

The model was run for a series of chemicals of log K_{ow} ranging from 3 to 8, including PCBs. The results are summarized in Table 3.3 and are discussed in sequence below. In the interests of brevity, the chemicals are referred to as chemicals 3, 4, 5, etc., corresponding to their log K_{ow} values.

Table 3.3. Effect of Varying K_{ow} on Sediment-Water Exchange Processes

log K_{OW}	3	4	5	6	6.6 (PCB)	7	8
Concentration in water (g/m^3)							
Total	1.14	1.14	1.14	1.14	1.14	1.14	1.14
Dissolved	1.14	1.14	1.13	1.09	0.98	0.81	0.22
Sorbed	0.0001	0.001	0.01	0.05	0.16	.33	0.92
Fugacity in water (nPa)	42.7	42.7	42.5	41.0	36.7	30.3	8.4
Fugacity in sediment (nPa)	43.1	46.9	78.5	182.5	20.7	181.1	52.0
Concentration in sediment μg/g	1.7×10^{-5}	1.8×10^{-4}	3×10^{-3}	7.1×10^{-2}	0.3	0.7	2.0
Ratio f_s/f_w	1.01	1.10	1.85	4.45	5.6	6.0	6.2
Biotic concentration (μg/g)							
In water	6×10^{-5}	6×10^{-4}	6×10^{-3}	0.05	0.19	0.4	1.12
In sediment	6×10^{-5}	6×10^{-4}	10×10^{-3}	0.24	1.10	2.4	6.94
Transfer rates (kg/year)							
Water-sediment transfer	78.7	87.0	170.4	971	3264	6678	18325
Deposition	0.9	9.3	93	897	3197	6623	18310
Diffusion	77.8	77.7	77.4	74	67	55	15
Sediment to water transfer	78.6	86.2	155.6	628	1712	3266	8525
Resuspension	.1	0.8	12.7	295	1335	2935	8430
Diffusion	78.5	85.4	142.9	332	377	330	95
Burial	.07	0.8	12.9	300	1353	2976	8546
Reaction	.01	0.1	1.9	44	199	436	1253
Mass in water (kg)	1908	1908	1908	1908	1908	1908	1908
Mass in sediment (kg)	0.7	7	108	2514	11347	25000	71680
Ratio s/w masses	0.0003	0.003	0.06	1.32	5.9	13.1	38
Residence times (years)							
Water	24	22	11	2	0.6	0.3	0.1
Sediment	0.01	0.1	0.6	2.6	3.5	3.7	3.9

Condition in the Water Column

The water column concentration of sorbing organic matter is assumed to be approximately 0.1 ppm; thus only when log K_{ow} exceeds 6 is there an appreciable fraction of the chemical sorbed to the suspended matter. Chemicals 3, 4, and 5 are almost entirely in solution, 6 is 5% sorbed, 7 is nearly equipartitioned, and chemical 8 is only 20% in solution. As a result, the water column fugacities of chemicals 3, 4, 5, and 6 are fairly constant, most chemical being in dissolved form and thus able to exert its full fugacity. The water column fugacity falls for 7, and more significantly for 8. The mass of chemical in the water column is a constant 1900 kg because of the imposed constant concentration.

The equilibrium concentration in water column biota (e.g., small fish) increases steadily and linearly with K_{ow} from negligible values for chemicals 3 and 4, to approximately 0.05 μg/g for 6, 0.4 μg/g for 7, and 1.1 μg/g for 8. There is thus a tendency for this increase in concentration to level off. Although the organism-to-water partition coefficient is steadily increasing, the fraction of the chemical available or "bioavailable" in the water column to accomplish this partitioning is decreasing. The high bioaccumulation levels expected with very hydrophobic chemicals are therefore offset significantly by competitive partitioning into particulate (and also colloidal) organic matter in the water column. In reality, there may be a kinetic limitation to partitioning of chemicals 7 and 8; thus, lower concentrations may be encountered. Further, as discussed by Thomann,[10] the food chain effect becomes more important than water-to-lipid partitioning; thus, this simple calculation could be misleading. It does, however, show the nature of the basic dependence of bioconcentration on K_{ow}, and on bioavailability as controlled by the organic matter content of the water column.

Condition in the Sediment

The sediment fugacities increase relatively slowly for chemicals 3, 4, and 5, with values similar to, but somewhat exceeding, those in the water column. The ratio of sediment-to-water fugacity rises from about 1 to about 1.8 because decomposition or mineralization of the deposited organic carbon increases the concentration of chemical in the remaining organic carbon, and thus causes an increase in the chemical's fugacity. Essentially the "solvent" is partially removed. If there was no mineralization of the organic carbon, the fugacity in the sediment would be approximately equal to that of the water. If the chemical were to react significantly in the sediment, the fugacity would be reduced. When DT is large compared to DD and DR (i.e., the chemical is less hydrophobic), diffusion tends to restore equi-fugacity between sediment and water. This raises an interesting point: in monitoring programs it may be valuable to examine the equilibrium status of the sediment relative to that of the water. This has been attempted by Murphy,[11] who has undertaken air stripping experiments to show that in lake systems the sediment and water fugacities are similar in magnitude.

It would be interesting to examine a wide range of lake sediment and water column data, to test the assertion that the fugacities are similar in magnitude, and to explore if there is some systematic variation in their fugacity ratios with parameters such as K_{ow} and organic carbon fate.

The steady-state sediment concentrations rise from negligible values for chemical 3 approximately in proportion to K_{ow}, but with a tendency to level off for chemicals 7 and 8. The mass of chemical in the sediment rises correspondingly; thus, the ratio of the mass of chemical in the sediment to mass in water is very small for chemicals 3, 4, and 5, and about equal for chemical 6, while for 7 and 8, the mass in the sediment greatly exceeds that in the water. If there is an in-place contaminant problem, the contaminant will be in-place in the water for chemicals 3, 4, and 5, and in the sediment for 6, 7, and 8. Since water is usually advected from the system fairly rapidly, except in very large lakes such as Lake Superior, the in-place pollution problem generally lies (as is intuitively obvious) in the sediment.

Chemical concentrations in the benthos track the concentrations in the sediment generally with a constant ratio, because in both benthos and solids the contaminant is dissolved in an organic carbon or lipid matrix, which have generally similar properties. This relationship between benthic and sediment concentrations has been noted by Connor[12] and discussed by Reuber et al.[8] The biota concentrations in the water track the fugacity in the water column and are generally similar in magnitude to those of the sediments, but because the fugacity effect in the sediment tends to exceed that in the water, the benthos usually have a greater contaminant concentration than water column organisms.

The results suggest gathering and analyzing data on water column and sediment biotic concentrations, preferably normalized on a lipid basis. Indeed, biotic concentrations may be the most reliable indicators of fugacities in the system, at least for chemicals that are not metabolized. Detailed examination of these fugacities by Connolly has shown that, in practice, most benthic organisms are at a somewhat higher fugacity than the sediment,[13] perhaps due to the consumption of organic carbon, which increases the contaminant fugacity by a simple concentration mechanism.

Recently Ferraro et al. have undertaken a comprehensive test of such a fugacity-based model and, in addition to providing an excellent review of this growing literature, have shown that the fugacity, or "accumulation factor," model gives a satisfactory description of the basic relationship between levels of sediment and benthic biota contamination.[14]

Water-to-Sediment Transfer

The calculated water-to-sediment transfer process rates suggest that diffusion rates are relatively constant for chemicals 3 to 6, whereas particle deposition increases greatly with K_{ow}. As a result of the preferential partitioning of chemical into these particles, the deposition rate tends to reach a maximum when virtually all the chemical in the water column is sorbed and is subject to particle deposition. Although water-phase processes are most important for low K_{ow} chemicals, particle transport becomes most important at high K_{ow} values, as was suggested earlier.

In this series of calculations a constant water column concentration was arbitrarily imposed on the system. If a constant chemical input rate had been imposed, the water column concentrations would have been very low for the high K_{ow} chemicals because of the efficient removal. In practice, it would be difficult to sustain a large water column concentration of a very hydrophobic chemical without very large emission rates, which would be equivalent to the large calculated deposition rates.

If the amount in the water column (kg) is divided by the transfer rates (kg/year), a clearance time is obtained. This is the time necessary for the removal processes to substantially deplete the water column of chemical. As is shown in Table 3.3, this is very long for chemicals 3, 4, and 5, but it becomes fairly short for chemicals 6, 7, and 8. The implication is that hydrophobic chemicals present in the water column will be appreciably removed by deposition, while more hydrophilic chemicals will tend to remain in the water to be removed by other processes such as evaporation, water advection, diffusion, and biodegradation.

The very short water residence times for chemicals 7 and 8 are unrealistic because deposition will be limited by the velocity at which particles can settle, i.e., about 1 m per day, and by the seasonal fluctuations in particulate organic carbon content of the water column. The equations contain the inherent assumption that conditions are well-mixed and constant throughout the year; thus, residence times of a fraction of a year are possible. In reality the seasonal fluctuations invalidate this simple assumption. The key point is that the model shows that for very hydrophobic chemicals, deposition will be controlled by the particulate organic carbon budget, not by K_{ow}.

Sediment-to-Water Transfer and Sediment Losses

Considering the reverse processes contributing to loss of chemical from sediment, the total rate must, of course, equal the rate of transport from the water. Diffusion in solution from sediment to water generally exceeds that from water to sediment because of the higher sediment fugacity, but the diffusive rates are only significant for low K_{ow} chemicals—3, 4, 5, and 6.

Transport of chemical to the water column in association with particulate organic carbon becomes more important as K_{ow} increases. This transport can occur either by direct resuspension of sediment solids or by diffusion of organic colloids containing the chemical. There is considerable uncertainty about these rates; however, it appears that they may be comparable in magnitude. Since both involve the same medium for transport, the ratio of resuspension-to-colloid diffusion rates should be fairly constant.

The burial and reaction terms generally involve the removal of chemical from a fixed proportion of the sediment volume per year. Thus, since most of the chemical is associated with the organic carbon, or at least with the solid phase, these rates increase in parallel with the resuspension rates. Here the ratio of rates are approximately resuspension, 8; burial, 8; reaction, 1; with diffusion being variable. It must be emphasized that, as discussed by Mackay,[3] the assumed reaction rate is purely illustrative, but it is believed to be reasonable in magnitude. Although constant reaction rates and burial rates are assumed here, the magnitude of the absolute rate (kg/year) increases significantly with K_{ow} because of the larger mass of chemical in the sediment that is susceptible to these processes. Thus, even very slow reactions, with half-lives measured in decades, can become very significant as removal processes for hydrophobic chemicals that are predominantly partitioned into the sediments, and remain there for periods of decades prior to burial.

Finally, the clearance time for chemical from sediment can be calculated as the ratio of the amount in the sediment to the transfer rate to, or from, the sediment. This time is very short for chemicals 3, 4, and 5 but increases to about 4 years for the more hydrophobic chemicals. This time is very sensitive to the assumed sediment depth. This reinforces the concept that sediment plays a relatively minor role for chemicals 3, 4, and 5 since its capacity for these chemicals is limited, and there is rapid transport of these chemicals back to the water column.

Some of these findings are consolidated in Figure 3.3, which shows the water-to-sediment and sediment loss processes. For chemicals 3, 4, and 5, which are primarily in a dissolved state, diffusion from water to sediment, and sediment to water, are the dominant exchange mechanisms, with most chemical remaining in the water column and the sediment playing a relatively minor role. For chemicals 6, 7, and 8, most chemical is transported in association with particulate organic carbon, and it is the solids' behavior that controls the chemicals' dynamics. Most chemical is associated with the sediment, and the water plays a relatively minor role. Processes in which the chemicals are transported in the water phase are unimportant. These deductions are, of course, sensitive to the ratio of water-to-sediment depths of the system (here 17200). It is when the sediment-water partition coefficient equals this number that the "switch" from hydrophilic water-dominated to hydrophobic sediment-solids-dominated behavior tends to occur. This ratio may be a useful descriptor of lake characteristics, varying from about 10^4 for deep lakes, to 10^2 for shallow lakes, and in the extreme to about 1.0 for wetlands or marshes. Resuspension, colloid diffusion, burial, and reaction each tends to contribute a constant proportion to the overall loss process. Throughout the range of chemical hydrophobicity, the fugacity of a conservative chemical in the sediment should be comparable to that in the water, and the biotic concentrations should show

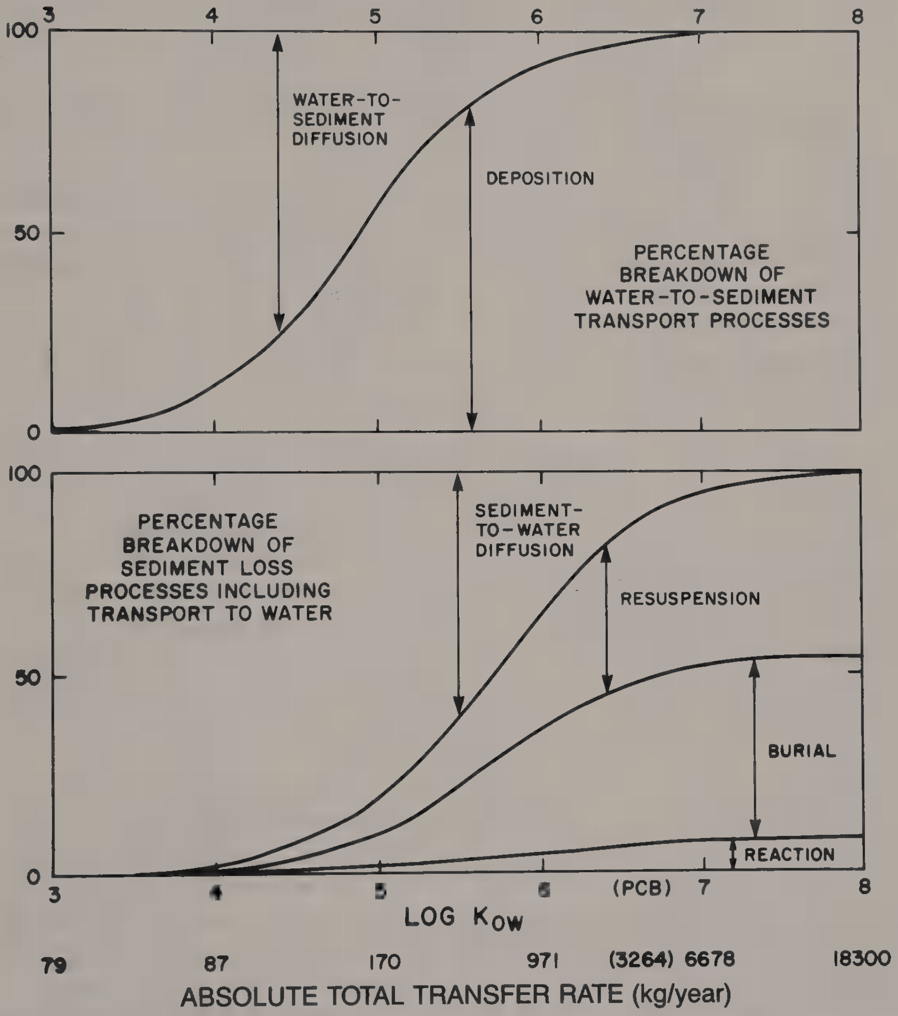


Figure 3.3. Breakdown of water-to-sediment transport and sediment-to-water transport and transformation processes at steady state under the conditions defined in Table 3.3.

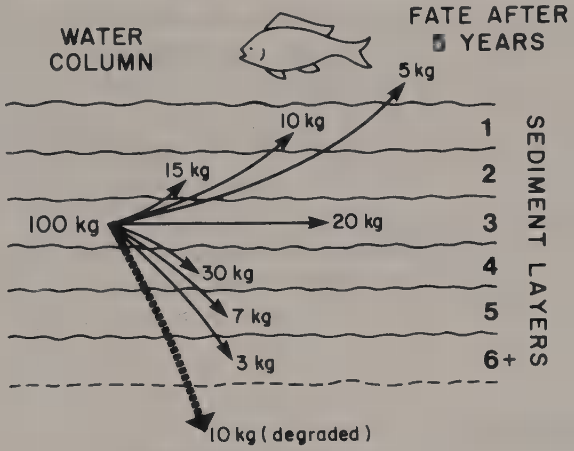
similar behavior, with a biomagnification effect of uncertain magnitude tending to increase biotic fugacities.

DISCUSSION

This analysis has demonstrated what are believed to be the likely general behavior patterns of chemicals as they exchange between the water column and sediment as a function of K_{ow}, or the related particle-to-water partition coefficient. Obviously, when there are relatively fast removal processes in the water column—for example, evaporation, biodegradation, or water flow, as may occur in rivers—these simple relationships will break down.

The principal weakness in this discussion is the assumption of a well-mixed sediment layer. Greater fidelity to actual conditions could be obtained by invoking multiple layers and defining rates of transport of pore water, sediment solids, and colloids between these layers. However, as more layers are introduced, the model becomes more complex, and there is a corresponding need for more transport and transformation rate data as a function of depth in the sediment. Studies of multilayer transport have been most valuable when examining sediment chronology, i.e., the nature of sediment concentration profiles as a function of depth or time of chemical entry into the aquatic system, as has been discussed, for example, by Charles and Hites,[15] Christensen and Goetz,[16] and for Lake Ontario by Eisenreich et al.[17]

In many respects, the problem of calculating the behavior of chemicals in sediment is similar to that of calculating the behavior of chemicals in soils exposed to the atmosphere. Transfer to the atmosphere by evaporation is strongly dependent on the depth of chemical incorporation into the soil. This has been discussed by Stiver and Mackay,[2] who suggested a procedure, essentially the one originally presented by Christensen and Goetz,[16] that it should be possible to use linear mathematical models to calculate the fate of unit concentration of chemical in each layer. This is depicted schematically in Figure 3.4, in which hypothetical times are suggested. For example, after 5 years, of the amount of chemical initially in layer 3, 5% will return to the water column, 10% will move to layer 1, 15% will rise to layer 2, 20% will remain in layer 3, 10% will degrade, and the remaining 40% will be conveyed to greater depths in layers 4, 5, and 6, largely as a result of burial. It can thus be argued that after 5 years the water column will be exposed to 5% of the chemical now present in layer 3. This figure will ultimately rise to, say, 8%. A similar analysis may show that 25% of chemical in layer 2 will return to the water. Chemical presently deeper than layer 4 may be essentially permanently isolated from the water column. By examining observed sediment profiles with such mass balance statements in matrix form, it may be possible to estimate the amount of chemical in the sediment that is, or will become, accessible to the water column. If the estimated exposure is too large, remedial actions such as dredging or capping may be desirable. Regrettably, there is a tendency for much of the excellent work on sediment transport chronology to be under-utilized for practical purposes because of the complexity of the mathematics involved and the problem of translating the results into readily assimilable form. We suggest that by presenting the results of these mathematical models in terms of simple unit responses in matrix form, it should be possible to facilitate and encourage the use of these data in support of regulatory decisionmaking.



	PRESENT	+5 YEARS	+10 YEARS	+15 YEARS
WATER COLUMN	—	5	7	8
LAYER 1	—	10	5	3
LAYER 2	—	15	10	7
LAYER 3	100	20	15	10
LAYER 4	—	30	17	
LAYER 5	—	7	23	
LAYER 6 AND LOWER	—	3	5	
DEGRADED	—	10	18	

FATE MATRIX FOR 100 kg OF CHEMICAL INITIALLY IN LAYER 3

Figure 3.4. Illustration of a mass balance fate matrix describing the future behavior of chemical presently in a layer of contaminated sediment. Similar matrices could be compiled for other layers and for various time periods. It is thus possible to estimate the effective depth at which contaminant becomes essentially inaccessible to the water column.

CONCLUSIONS

In this chapter, we have first suggested that by having available a reliable, simple, linear model of an aquatic system, it may be possible to discriminate between sources of chemical contamination, such as atmospheric deposition, direct discharges, and advective inflow, and also to quantify the contribution from in-place pollution. It is believed that by using this approach, the regulatory decisionmaking process can be improved. Second, by exploring the fate of a range of chemicals in an evaluative lake, certain generalizations of contaminant behavior emerge. Most important is the assertion that for low K_{ow} chemicals, water-phase processes are most important; for high K_{ow} chemicals, the fate of particulate organic carbon is critical. An intermediate range exists in which both processes are important. It is suggested that examining the equilibrium status of chemicals (as a supplement to concentrations) present in water, sediment, and their biota has the potential to bring enhanced insights into chemical behavior in these important, and still inadequately understood, systems. Finally, there is an incentive to develop a deeper understanding of the fundamental chemical dynamics in sediment as a function of depth in order that the major weaknesses imposed by assuming a single well-mixed layer to apply can be remedied. These depths and dynamics ultimately control the capacity of sediment to retain chemicals or to return them to recontaminate the overlying aquatic ecosystem.

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CHAPTER 4

Application of Biotechnology to Water Quality Monitoring

Ivor T. Knight and Rita R. Colwell

INTRODUCTION

The fields of molecular genetics and, in particular, recombinant DNA technology have enabled the development of new approaches to environmental monitoring. One area of research is the use of DNA and RNA hybridization probes to identify contaminating microorganisms in water systems. Probes that are specific for selected indicator organisms or pathogens have already been produced and are being tested for use in determining the microbiological quality of drinking, fishing, and recreational waters. A second approach is the use of genetically engineered microorganisms as biosensors to indicate the concentration of toxic contaminants in a given environment more sensitively than can be done by traditional chemical methods and instrumentation. Both of these approaches employ the novel tools of molecular biology to improve both the specificity and sensitivity of water quality assays. This overview discusses both the theory and practice of these approaches and evaluates their application to future water quality monitoring methodology.

HYBRIDIZATION PROBES

The basis of the hybridization probe assay is the ability of complementary single strands of nucleic acid to form stable, double-stranded structures via base pairing. The reaction, commonly termed *renaturation* or *annealing,* can occur between two strands of DNA, two strands of RNA, or between a strand of RNA and a strand of DNA. Since renaturation results in hydrogen bonds between complementary bases in each strand, conditions that affect hydrogen bonding, such as temperature and ionic strength, can influence the specificity of the reaction. If, for example, hybridization is performed under conditions that approach those in which the hybrid is unstable, then only highly complimentary strands will form stable hybrids. These conditions are referred to as high stringency conditions because they require that both strands be well matched for them to anneal. Low stringency hybridization conditions are achieved by lowering the temperature and raising the concentration of monovalent cations and result in formation of hybrids between strands which are not as well matched.

Nucleic acid probes are molecules of single-stranded DNA or RNA that have been labeled, either chemically or radioactively, so that they can be detected in a mixture of renatured nucleic acids. The probe is incubated with denatured test DNA or RNA (often total cellular DNA) and, under appropriate conditions, hybridizes with regions of the test nucleic acid that are complementary (target sequence). Excess, unhybridized probe is selectively removed and probe:target hybrids are detected using methods appropriate to the particular labeling strategy. If, for example, the probe is radioactively labeled, it can be detected by autoradiography or liquid scintillation counting. Nonradioactive labels are generally detected using a color development system.

The most common format for the probe hybridization assay is the mixed-phase or membrane hybridization assay, pioneered by Southern.[1] Test DNA or RNA is denatured and allowed to bind to nitrosylated cellulose membrane (nitrocellulose), a nylon-based membrane, or a membrane composed of a nylon-nitrocellulose mixture. These membranes bind single-stranded nucleic acids reversibly, but by heat treatment or by exposure to ultraviolet light the DNA can be linked covalently to the membrane, with the target sequence available for hybridization with probe nucleic acid. After incubation with the probe (hybridization), excess unhybridized probe is washed from the filter, and the filter is examined for presence and amount of hybridized probe. Discussion of the theory and practice of mixed-phase hybridization is provided by Meinkoth and Wahl.[2]

DNA and RNA hybridization probes offer a means of circumventing one of the acute problems encountered in the assessment of the microbiological quality of water, namely, determining the degree of contamination by pathogenic and indicator microorganisms which cannot be easily cultured in the laboratory. Since present microbiological analyses rely upon cultivation procedures before a determination is made,[3] organisms that grow poorly or cannot be cultured will be grossly underestimated. This is especially a problem for enumeration of viruses, but can also be a significant problem for bacteria, as well.

The efficacy of culturing methods for detection of bacterial pathogens from water samples is complicated by the nonculturable stage and "sublethal injury" phenomena, both of which have been described for many enteropathogenic bacteria found in the aquatic environment. The nonculturable stage is part of a survival strategy, described by Roszak and Colwell,[4] whereby bacteria, including many enteric organisms, respond to a transition from nutrient-rich, stable environments (such as an animal gut or a laboratory culture) to a less supportive environment (such as estuarine water) by entering a dormant stage. The response is observed as a state in which the organism is viable, as measured by substrate uptake assay, but fails to grow in culture.[4] This phenomenon has been observed for several waterborne pathogens, including *Salmonella enteriditis*,[5] enterotoxigenic *E. coli, Vibrio cholerae, Shigella* spp.,[6,7] and *Campylobacter jejuni*.[8] "Sublethal injury" of enteropathogenic bacteria has been described by Singh and McFeters as sublethal cellular lesions and alterations in physiology following exposure to aquatic stress factors which result in a temporary reduction or loss of virulence and reduced ability to grow on selective media.[9] Stressors, such as chlorine and copper ions, in water supply and distribution systems have been shown to induce sublethal injury in several enteropathogenic bacteria.[10]

The application of nucleic acid probes to the detection of enteropathogenic microorganisms in water offers a means of eliminating the necessity of isolating specific pathogens in culture, prior to identification and enumeration. Many pathogen-specific probes have already been constructed and are being tested for use in water quality analysis.

Assays that use hybridization probes vary in their sensitivity, depending upon the type of probe used and the labeling and detection system employed to measure the amount of probe which is hybridized to the target sequence. In general, however, the lowest amount of target DNA required is from 0.1 to 1 pg. If the target is present only once per organism, then the minimum number of organisms required for a positive signal may be as many as 10^6. Methods that increase target concentration are presently an active area of research.

Two approaches have proved successful thus far. The first is sample concentration prior to nucleic acid extraction, and the second is specific target amplification using an in vitro enzymatic method called the polymerase chain reaction (PCR). One example of the former approach is the method developed by Somerville et al., which uses a high capacity disposable membrane filtration unit to concentrate the microflora in aquatic samples for extraction of DNA and RNA.[11] The method is capable of extracting the microbial DNA and RNA from 10-L samples in an efficient, cost-effective manner. This method, developed in our laboratory, was used to recover DNA from estuarine samples for the application of a *Salmonella*-specific hybridization probe assay.[12]

PCR, described by Mullis and Faloona,[13] is a technique for amplifying discrete fragments of DNA from complex mixtures and has recently been improved by Saiki et al. to provide a simple method for increasing target DNA concentration over a billionfold.[14] The method is called polymerase chain reaction because DNA polymerase is used in conjunction with primers which flank the target sequence to synthesize nascent DNA between the primers. The nascent DNA then serves as a target for subsequent rounds of polymerization, resulting in a geometric increase in concentration of the target sequence. This technique has been incorporated into a DNA probe assay for detection of fecal coliform bacteria in drinking water[15] and for specific detection of enterotoxigenic bacteria.[16] Although PCR is a powerful method for enhancing the detection of rare targets in DNA samples, it has the disadvantage of making the probe assay qualitative rather than quantitative, since amplification does not proceed at a constant rate over the entire course of the reaction.

While the use of DNA and RNA probes for analysis of water quality is an active area of research, there are several hurdles that must be overcome before hybridization probes are used routinely in testing laboratories. The technology is more expensive than standard methods and employs more sophisticated techniques, and so requires a higher level of training in personnel. Also, radioactive labeling systems, with their attendant safety hazards, remain the method of choice for hybridization assays. Although nonradioactive labels are improving to the point where their sensitivity rivals that of radioactive labels, there are still problems of cross-reactivity arising from colormetric detection systems that must be solved before routine application of nonradioactive probes can be considered. Simplification of the probe assay to reduce the cost and training necessary to conduct them and the development of reliable nonradioactive detection systems are, however, areas of active research. Private firms, hoping to market probe-based detection kits, are especially active in resolving the problems and making probe assays accessible to testing laboratories. In the near future there will likely be reliable, nonradioactive probe-based detection kits on the market for determining the microbiological quality of water.

BIOSENSORS

Another area where recombinant DNA technology will impact on environmental testing is in the development of microorganisms that are genetically engineered to respond to specific toxicants by producing a response that can be measured by conventional instrumentation. These organisms are called *biosensors* and can be much more sensitive than conventional quantitative techniques.

The ability to construct such genetically engineered microorganisms (GEMs) is derived, in large part, from basic research into the molecular genetics of catabolic phenotypes in bacteria. Research on the genes controlling naphthalene degradation by bacteria has provided the basis for the most recent breakthrough in this field, the construction of a naphthalene biosensor that employs a bioluminescent reporter gene.[17] This biosensor was constructed by modifying a naturally occurring bacterium that degrades naphthalene. It was modified by inserting bacterial luciferase (*lux*) genes, which encodes light production, into a gene in the naphthalene catabolic pathway (*nahG*) in such a way that the expression of the *lux* gene is regulated by the same elements that control the *nahG* gene. Normally, when naphthalene-degrading bacteria are growing in the presence of naphthalene, the *nahG* gene produces salicylate hydrolase, an enzyme that breaks down an intermediate in the naphthalene catabolic pathway. In the modified bacterium, the same regulatory elements now control the *lux* genes, and light is produced in the presence of naphthalene. This light can be measured using a photomultiplier detection system. The developers of the naphthalene biosensor have demonstrated that the luminescent response to naphthalene in sediments is rapid and concentration-dependent, and they make the case that this technology could be applied to on-line monitoring of groundwater supplies.[17]

Bacterial degradation and transformation of toxic substances is an active area of research, and as the molecular genetics of these processes is elucidated, one can expect construction of more recombinant bacteria for use as biosensors.

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PART III

UPTAKE AND ACCUMULATION OF SEDIMENT—ASSOCIATED CONTAMINANTS

A. Bioavailability

CHAPTER 5

A Clam's Eye View of the Bioavailability of Sediment-Associated Pollutants

Henry Lee II

INTRODUCTION

Bioavailability of a pollutant is one of the key factors controlling its bioaccumulation and toxicity. For a truly dissolved pollutant, bioavailability can be considered as how readily an organism accumulates a pollutant from the surrounding water. The concept of bioavailability is more difficult to apply to sediment-associated pollutants, and the term is often used without an exact definition. This difficulty is related both to the presence of multiple sources of sediment-associated pollutants (e.g., interstitial water, particulates) and to the ability of sediment-dwelling organisms to modify their exposure by manipulating their local environment. However, without a rigorous definition it is difficult to assess the importance of the chemical and biological factors regulating sediment bioavailability or to quantitatively compare bioavailability among compounds, sediments, or organisms.

This chapter proposes a definition of bioavailability for sediment-associated pollutants and outlines the approaches to quantifying sediment bioavailability. The chapter then presents an overview of the ecological and physiological processes potentially affecting bioavailability. This overview draws heavily on our work with polychlorinated biphenyls (PCBs) and hexachlorobenzene (HCB) with the marine deposit-feeding clam *Macoma nasuta*. One of the main points is that standard measurements of sediment pollutant concentrations may not adequately represent an organism's exposure.

DEFINITION AND MEASUREMENT OF THE BIOAVAILABILITY OF SEDIMENT-ASSOCIATED POLLUTANTS

Definition of Sediment Bioavailability

In a general sense, a pollutant can be considered bioavailable if it is transferred from the sediment milieu into an infaunal organism. The greater the transfer, the greater the bioavailability. To avoid confounding effects, the measure of bioavailability should include only factors directly related to the transfer of the contaminant. At least four measures of sediment bioavailability have been used or implied in the literature: steady-state body burdens, toxicity, efficiency of pollutant uptake, and the rate of pollutant uptake.

Steady-state body burdens are a function of an organism's ability to depurate/metabolize the parent compound as well as the uptake of the parent compound. The rate of depuration and metabolism vary substantially among species and can vary with tissue residue (e.g., induction of enzymes). It is possible for two species to have similar uptake rates but substantially different steady-state body burdens because of differences in elimination rates. For this reason, steady-state body burdens are not a suitable absolute measure of bioavailability. However, steady-state tissue residues can be used as a comparative measure of the relative bioavailability if the elimination rate can be assumed to be equal, such as when the same species is exposed to different sediment types.

Similarly, sediment toxicity as a measure of bioavailability is confounded by a whole suite of physiological-biochemical processes. Because toxicity may vary with the rate of accumulation as well as with the amount accumulated and the physiological-biochemical process may vary among sediment types, toxicity should be used with caution even as a comparative measure of sediment bioavailability.

The third potential measure is how efficiently the organism extracts (or assimilates) the pollutant from the sediment. Such a measure would be analogous to the efficiency of gill uptake used to measure the relative bioavailability of dissolved pollutants in fish.[1,2] However, as opposed to dissolved pollutants, there are several potential sediment uptake routes with different sites of accumulation (e.g., interstitial water at gills, ingested sediment particles in gut) making it impossible to assign a single efficiency value to the entire sediment.

The last potential measure is the rate transfer of a pollutant from the sediment milieu to an organism. Defining bioavailability as the rate of transfer or uptake from the entire sediment integrates uptake from the various pollutant pools, thereby avoiding the problem associated with the efficiency measurements. At least to a first approximation, the rate of uptake is assumed to be independent of the tissue residue,[3,4] thereby avoiding or at least reducing the problem of confounding uptake and elimination processes. This rate needs to be normalized by organism weight and sediment pollutant concentration to account for differences in organism size and pollutant concentration, respectively. Therefore, sediment bioavailability becomes the weight-specific increase in tissue residues per unit time normalized to the sediment pollutant concentration:

$$\frac{dCt/dt}{C_s} = \frac{\mu g/(\text{g tissue} \times \text{time})}{\mu g/(\text{g sediment})} \tag{5.1}$$

or

$$K_s = (\text{g sediment})/(\text{g tissue} \times \text{time})$$

where C_t = tissue residue on weight-specific basis (μg/g tissue)
C_s = sediment pollutant concentration (μg/g sediment)
t = time
K_s = sediment uptake rate coefficient, or uptake clearance

K_s can be referred to as the *sediment uptake rate coefficient*, or the *uptake clearance* as used in Landrum.[5] It is referred to as a "coefficient" rather than a "constant" because the value for a compound is not fixed but can vary with physical and biological factors. The term *uptake clearance* comes from pharmacokinetics and in this case represents the amount of sediment cleared (stripped) of pollutant per gram of tissue per unit time. Uptake clearance is not a concept used widely in aquatic toxicology, though the use of pharmacokinetic nomenclature would promote comparisons between toxicokinetic and clinical pharmacokinetic models.

If the grams are canceled in Equation 5.1, K_s collapses to 1/time, or the *uptake rate constant*, k_1. Uptake rate constants derived from water exposures are frequently used in predicting water uptake,[3,4,6] and the use of k_1's derived from sediment exposures have been suggested as a way to predict steady-state body burdens in infaunal organisms.[7] However, as has been pointed out (Stehly et al.[8] and P. Landrum, personal communication), the grams should not be canceled and the appropriate units are g/(g × time) for sediment and mL/(g × time) for water exposures. This does not change the numerical value but helps avoid confusing rates derived from a water exposure versus a sediment exposure. Maintaining the units also reveals that the value of K_s will vary depending on whether the tissue and sediment concentrations were in dry or wet weight units. K_s's derived from wet and dry weight concentrations can vary several fold, so it is important to define the units used. It is also important to emphasize that this definition of bioavailability does not imply that a K_s for a compound is necessarily constant either among sediments or species.

Measurement and Comparison of Sediment Bioavailability

The K_s for a particular sediment type for a particular species can be determined by dividing the slope of the rate of increase in tissue residues (μg/g tissue × time) measured during the linear uptake phase by the sediment concentration (μg/g sediment) (Figure 5.1). A good fit to a linear regression indicates the uptake was in the linear uptake phase; a poor fit suggests that the exposure duration extended past the linear uptake phase. A K_s derived from a nonlinear uptake curve, where elimination is nontrivial, will underestimate the true K_s. Figure 5.1 shows the uptake of three PCB congeners over ten days by *Macoma* (work in progress). Both 2,2′,5,5′-tetrachlorobiphenyl (IUPAC #52) and 2,2′,4,4′,5,5′-hexachlorobiphenyl (IUPAC #153) showed a highly significant linear regression, and the K_s can be calculated from the slope. However, for 2,2′,5-trichlorobiphenyl (IUPAC #18) the regression is not significant, presumably because this lower K_{ow} compound approaches steady-state within a few days.

The rate of uptake can also be estimated from a single tissue residue as long as the single sampling period is within the linear uptake phase. For the 11 PCB congeners and HCB that fit the linear regression, the average difference between the K_s calculated from the linear regression and the K_s calculated from

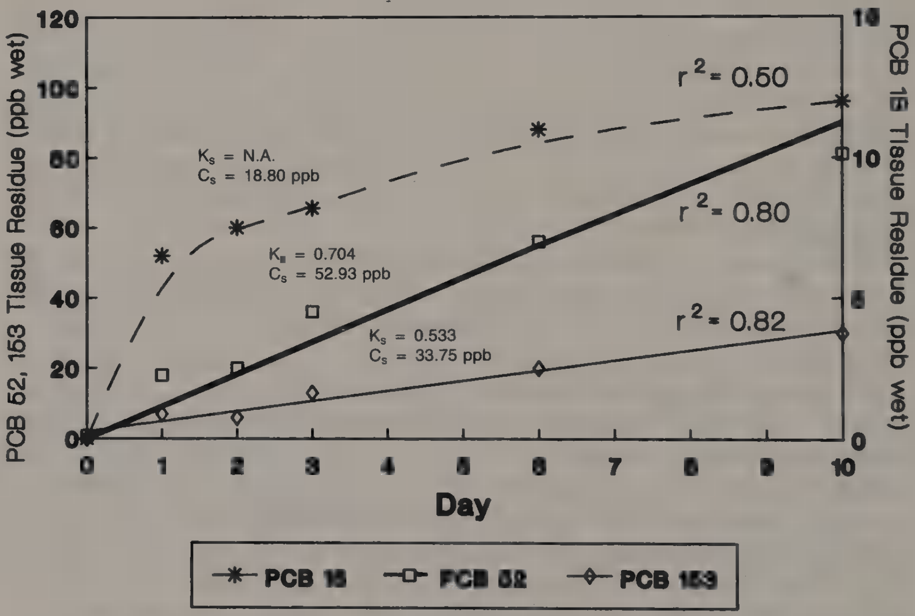

Figure 5.1. Calculation of K_s from the uptake rate measured in the linear uptake phase. K_s was calculated by dividing the slope of the linear regression of tissue residues versus time (μg/g tissue × day) by the sediment pollutant concentration (C_s = μg/g sediment). Both 2,2′,5,5′-tetrachlorobiphenyl (IUPAC #52) and 2,2′,4,4′,5,5′-hexachlorobiphenyl (IUPAC #153) showed a significant linear regression. 2,2′,5-Trichlorobiphenyl (IUPAC #18) had a nonlinear uptake curve over 10 days, so calculation by this method is not appropriate. All values are for *Macoma nasuta*, and the clams were not purged before chemical analysis. Sediment concentrations are in ppb dry weight, and the tissue residues were converted to dry weight before calculating K_s.

the 10-day tissue residue was only 3.3%. Use of the single point estimation approach allows calculation of K_s from published bioaccumulation tests that used a single exposure period, but the technique has to be applied with some knowledge of the elimination rate of the compound. K_s can also be calculated by fitting of nonlinear equations if the uptake is nonlinear (i.e., extends past linear uptake phase) or the sediment pollutant concentration varies.[5,8,9] However, the direct measurement of K_s during the linear uptake phase is favored because it avoids the possibility of nonexact solutions to the nonlinear equations and requires fitting only one parameter, which should generate a more accurate estimate for K_s.

Defining sediment bioavailability as K_s allows quantitative comparisons among studies. For example, Figure 5.2 shows the K_s's for 14 chlorinated compounds using *Macoma* (work in progress), the K_s's for several PAHs, and a PCB congener using the freshwater amphipod *Pontoporeia hoyi*,[5] and K_s's

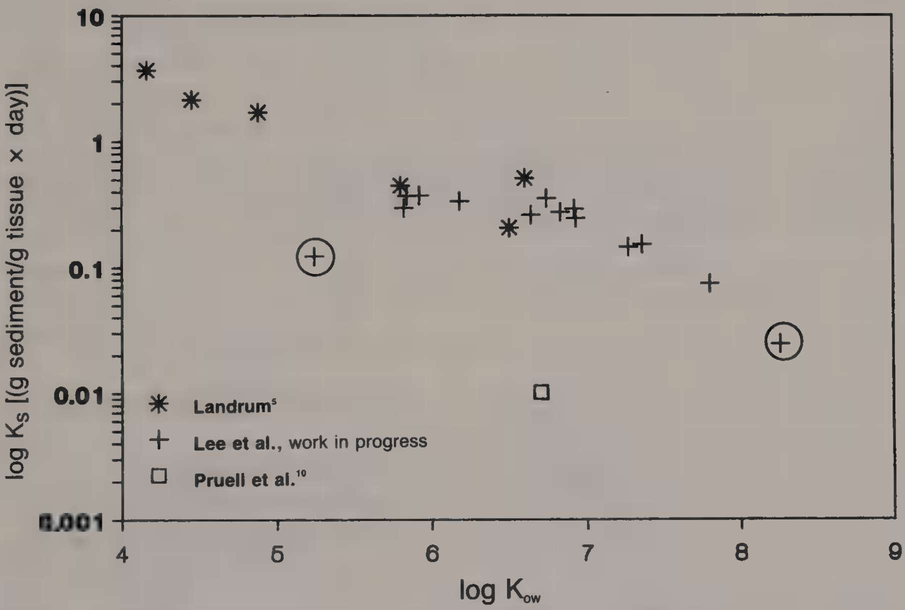

Figure 5.2. Log K_S as a function of log K_{ow}. Data from three different studies are plotted to show how expressing sediment bioavailability as K_S allows quantitative comparisons among studies. In all cases, the K_s's were calculated using dry sediment and tissue pollutant concentrations. Data from Lee et al. (work in progress) are for 13 PCB congeners and HCB using *Macoma nasuta*. The inclusion of PCB congeners 18 and 209 (⊕), which did not fit the linear uptake curve, is for illustrative purposes. TOC of the sediment was 1.3%. Data from Landrum are for 5 PAHs and ■ PCB using the freshwater amphipod *Pontoporeia hoyi*.[5] TOC of the sediment was 1.3%. Data from Pruell et al. are for 2,3,7,8-TCDD and total PCBs using *Nereis virens*, *Macoma nasuta*, and *Palaemonetes* sp.[10] All six TCDD and PCB values are represented by the single point. TOC of the sediment was 5.7%

for 2,3,7,8-TCDD and total PCBs for three estuarine species, including *Macoma*.[10] The values for *Pontoporeia* were derived using nonlinear equations, while the values for TCDD and total PCBs were calculated from the tissue residues at 10 days with residues at later sampling dates demonstrating that the organisms were still in the linear uptake phase. All values were converted to standard units (dry weight concentrations and day^{-1}). From Figure 5.2, it is possible to speculate that K_s declines with both log K_{ow} and TOC. A more detailed comparison is required before any specific conclusions can be drawn, and the main point of this comparison is to demonstrate that expressing bioavailability as K_s allows quantitative comparisons among studies using different sediments, organisms, and estimation techniques.

Toxicokinetic Representation of Sediment Bioavailability

K_s integrates uptake from all sediment phases and is a measure of the overall bioavailability of a compound in a particular sediment. While this is a required characteristic of a general measure of sediment bioavailability, K_s offers little insight into the routes of uptake. It is theoretically possible to have the same bioavailability from two sediments even though interstitial water was the dominant uptake route in one case and ingested sediment in the other. However, by using a bioenergetic-based toxicokinetic model,[11] it is possible to dissect this integrated measure into the actual processes regulating the pollutant transfer. Modifying the bioenergetic-based model for sediment uptake,[12] the rate of change in tissue residues in a sediment-dwelling organism can be modeled as

$$dC_t/dt = \Sigma(F_X C_{PX} E_{PX}) - E \tag{5.2}$$

where C_t = tissue residue on weight-specific basis (μg/g)
t = time
F_X = weight-specific flux of phase X through organism (g/g tissue × time)
C_{PX} = pollutant concentration in phase X (μg/g)
E_{PX} = extraction efficiency of pollutant in phase X (unitless)
X = phase to which the organism is exposed (W_o = overlying water; W_i = interstitial water; S = sediment; F = food)
E = elimination rate of pollutant due to depuration of parent compound and metabolic degradation (μg/g tissue × time)

Normalizing for sediment concentration and assuming the uptake is in the linear uptake phase so that the elimination rate is trivial (i.e., E approaches 0), the equation becomes

$$\frac{dC_t/dt}{C_s} = \frac{\Sigma(F_x C_{px} E_{px})}{C_s} = K_s \tag{5.3}$$

This equation identifies the two general processes regulating sediment bioavailability. The first is the organism's exposure, the mass of pollutant actually coming into contact with the organism. In terms of Equations 5.2 and 5.3, an organism's exposure is the product of the flux of water, food, or sediment (F_X) times the concentration in the phase (C_{PX}). Exposure is used here only on the microhabitat scale, the scale at which organisms can affect the flux (F_X) or concentration (C_{PX}) of a pollutant. These processes are intrinsic characteristics of each species and, hence, of a species' propensity to bioaccumulate. Of the pollutant coming into contact with the organism, only a portion of it is transferred from the environment across the organism's membranes. Therefore, the second process regulating bioavailability is the fraction of the pollutant extracted (or assimilated) by the organism. These are the E_{PX} terms in Equations 5.2 and 5.3, with each route of uptake having its own efficiency term.

The remainder of the chapter will explore how organisms can potentially modify their exposure to sediment pollutants or the transfer of the pollutant from the environment to the organism.

EXPOSURE PROCESSES

By living in a solid matrix, infaunal organisms are able to modify their local environment, and hence their pollutant exposure, to a much greater extent than water column organisms. As a result, an infaunal organism may be exposed to substantially different interstitial water pollutant concentrations (C_{PWi}) or particle pollutant concentrations (C_{PS}) than measured by standard sampling and chemical procedures.

Exposure to Sorbed Pollutants

Ingested sediment particles are the dominant uptake route for several high K_{ow} organic pollutants for certain organisms.[5,12,13] Therefore, the effects of feeding behavior on the flux of sediment (F_S) or pollutant concentration of the ingested particles (C_{PS}) may substantially affect sediment bioavailability. One important behavior is the organism's feeding zone. Deposit feeders show a wide variety of feeding types.[14] At one extreme, surface deposit feeders such as *Macoma* feed primarily on the upper few millimeters of sediment. At the other extreme, "conveyor-belt" species, which are head down and anus up in the sediment, ingest particles as deep as 20–30 cm below the surface. In sediments with distinct sediment horizons, these organisms would be exposed to substantially different pollutant concentrations than surface feeders (Figure 5.3).

Although the depth distributions in Figure 5.3 are hypothetical, dramatic differences in pollutant concentrations have been documented within the potential feeding range of deposit feeders. For example, the concentration of DDT has been as much as an order-of-magnitude greater at 20-cm depth than

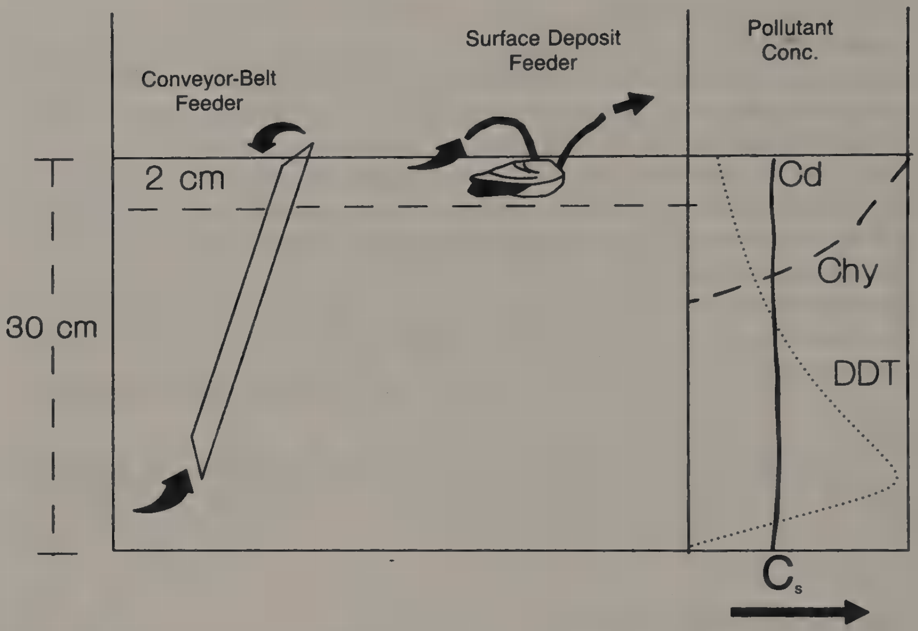

Figure 5.3. Effect of feeding depth on pollutant exposure. The conveyor-belt and surface deposit feeding modes illustrate the range in feeding depth by deposit feeders. The "standard" sediment collection depth of 2 cm for pollutant analysis is illustrated by the dashed line. The variations in sediment concentrations (C_s) of Cd, DDT, and chrysene (Chy) with depth are hypothetical but within the ranges reported for various sites. In this scenario, the standard sediment collection techniques would not adequately represent the exposure regime of either feeding type.

in the surface sediment on the Palos Verdes Peninsula.[15] PCBs also tend to be two- to threefold higher in the deeper sediments off of Palos Verdes.[16] These higher concentrations in deep sediment reflect the historical discharges of these chemicals. In other sites where there is a recent pollutant input or where there is an input of a rapidly degraded pollutant, the higher concentrations may be in the surface sediments. In these cases, the standard technique of sampling the upper few centimeters of sediment for pollutant analysis could substantially underestimate or overestimate an organism's exposure.

An aspect of deposit-feeding behavior that has been largely ignored in terms of pollutant exposure is selective feeding. Most deposit feeders selectively ingest the finer, higher TOC particles while discarding the larger, low TOC particles. This behavior can concentrate the organic content of the ingested sediment by more than an order-of-magnitude over that of the parent sediment. Using the 23 measurements in Cammen,[17] organic enrichment in the gut compared to the sediment averaged about 5-fold. Excluding the 61-fold increase reported for a deposit-feeding crab reduced the mean to a 2.4-fold organic carbon enrichment.

Because the sorption of neutral organic pollutants is directly related to the carbon content of the solids,[18,19] the pollutant concentration on the high TOC ingested particles should be correspondingly greater than that of the parent sediment. This was found with *Macoma,* in which the HCB concentration and the TOC of the ingested sediment was about two- to fourfold greater than the parent sediment concentration.[20] The greatest relative increase in pollutant concentration in the ingested sediment should occur in sandier sediments, which offer a relatively greater opportunity for selection of high TOC particles, and with compounds with high organic carbon partitioning coefficients (K_{oc}). However, the effect is not limited to neutral organics, as indicated by the 1.2- to 1.7-fold enrichment in the metal concentrations in the feces of deposit feeders.[21]

As a consequence of selective feeding, the pollutant concentrations measured in the parent sediment underestimate the actual dose ingested by selective deposit feeders. In essence, the organisms are "seeing" a more polluted environment than measured by standard techniques. Normalization of sediment pollutant concentrations to an organic carbon basis will partially account for this selection. This normalization assumes the carbon measured chemically is the same as that enriched in the ingested particles. This assumption is likely to be incorrect at least for species that select particles on the basis of protein levels.[22] Also, carbon normalization would not account for any processes related to the total pollutant concentration rather than carbon concentrations nor for processes dependent upon the volume or the volumetric flow of food, such as uptake by the GI tract.[23] Nonetheless, expressing pollutant concentrations on an organic carbon basis helps correct for the biological enhancement of neutral organics.

Exposure to Interstitial Water

Pollutant concentrations in interstitial water are normally many times higher than those in the overlying water. With these relatively high concentrations, interstitial water has the potential of being an important uptake route; Adams suggests that interstitial water is the dominant uptake route for neutral organics with a log K_{ow} of less than about 5.[24] In support of this suggestion, several experimenters have concluded that interstitial water was the dominant uptake route for certain neutral organics and heavy metals.[25,26] As with the ingested solids, biological processes affecting either the flux of the interstitial water (F_{Wi}) or the pollutant concentration in the interstitial water (C_{PWi}) can have a substantial impact on sediment bioavailability.

One of the key processes affecting exposure to interstitial water is the relative proportion ventilated compared to the amount of overlying water ventilated. The ventilation of interstitial water (F_{Wi}) varies both with the feeding type and the burrowing behavior of the organism. Infaunal filter feeders, such as *Mercenaria* and *Mya,* feed by filtering particles from the overlying water and so ventilate little interstitial water. Likewise, surface deposit-feeding bivalves, such as *Macoma*, ventilate an insignificant amount of interstitial water.[27] For these organisms, the only significant exposure to interstitial water pollutants is by sorption of dissolved pollutants to the integument. Passive sorption does not appear to be a major uptake route, at least for *Macoma* exposed to HCB.[12] At the other extreme, free-burrowing amphipods and polychaetes ventilate interstitial water exclusively (at least while buried in the sediment). Assuming that interstitial water concentrations approach the equilibrium concentrations established by the compound's K_{oc} and the TOC of the sediment, interstitial water could be a major uptake route for these organisms.

Dilution of Interstitial Water Pollutants

The assumption of equilibrium pollutant concentrations in the ventilated interstitial water would overestimate exposure if pollutant concentrations in the microhabitat surrounding the free-burrowing species are reduced by organismal uptake and/or advection of overlying water. The importance of depletion by organism uptake can be approximated from the rate of aqueous phase uptake (i.e., $F_{Wi}C_{PWi}E_{PW}$). The depleted pollutants can be renewed by desorption, diffusion, movement of the organism, advection of interstitial water, and excretion of the parent compound. To take the simplest case, let's assume that desorption is the only renewal mechanism, so the desorption rate has to equal or exceed the rate of aqueous-phase uptake to maintain the equilibrium interstitial water pollutant concentrations.

Using *Macoma* as an example, it is possible to estimate the volume of interstitial water potentially depleted. The uptake rate for HCB averaged about 0.008 μg/(g wet tissue × day) for *Macoma* (work in progress). *Macoma* extracts about 65% of the dissolved HCB,[28] and HCB has a water solubility of about 6 μg/L. Applying these values to a free-burrowing species and making a worst case assumption that all the uptake is from interstitial water, a 1-g organism would totally deplete about 2 mL of interstitial water per day. This is a relatively small volume and presumably a moderate desorption rate would maintain the concentration close to the equilibrium concentration. For very low solubility compounds (e.g., 2,3,7,8-TCDD = 0.2 ppb), a proportionally greater amount of interstitial water would be depleted for the same uptake rate. Therefore, it appears possible that biological uptake could deplete interstitial water concentrations, but that such effects would be limited to very low solubility pollutants.

The potentially more important process is the advection of overlying water diluting interstitial water concentrations. The volume of overlying water advected into the surface oxic sediment layer can be estimated from the renewal of interstitial oxygen. With no connection to the surface, free-burrowing forms (e.g., *Rhepoxynius*) meet their oxygen requirements by ventilating interstitial water and therefore have to spend the majority of their time in the oxic sediment layer. As there is no significant within-sediment generation of oxygen, the amount of oxygen advected into the sediment equals the total community respiration rate (sum of macrofaunal and microbial respiration and chemical oxygen demand) under steady-state conditions. Figure 5.4 is a representation of the process and shows a simplified method of estimating the minimum number of turnovers of the interstitial water in the oxic layer required to maintain an equilibrium oxygen concentration. Based on the values in Figure 5.4, a minimum of about 84 turnovers of the interstitial water per day are required. At this rate of 3–4 turnovers per hour, dilution of the interstitial water with overlying water may exceed the desorption rate of many pollutants. Admittedly, these calculations are oversimplifications, but they raise the possibility that infaunal organisms in the oxic layer may be exposed to interstitial water concentrations substantially less than equilibrium concentrations. If dilution is important, predictions of toxicity or bioaccumulation from equilibrium interstitial water concentrations will be overestimated. Also, estimates of the importance of interstitial water as an uptake route by applying bioconcentration factors (BCFs) to interstitial water may be overestimated because the concentration of the interstitial water in the microhabitat is overestimated. By overestimating the interstitial water route, such calculations would underestimate the importance of uptake from ingested sediment. These suggestions need to be tested by carefully sampling the interstitial water in the sediment layer inhabited by the free-burrowing organisms.

Tubes and Burrows

Another behavior affecting exposure to interstitial water is tube or burrow construction. Tubes and well-defined burrows are common among polychaetes, amphipods, and many other infaunal taxa. Tube/burrows are constructed of a polysaccharide inner lining and an outer layer of sediment of modified mineralogy, often with a higher TOC (Figure 5.5).[29,30] There is no definitive difference between a tube and a burrow, but in general a tube has a more defined structure whereas a burrow is the modification of the surrounding sediment. For the purposes of this discussion they are treated together.

Tubes and burrows vary greatly in their size, shape, and amount of water fluxed through the tube/burrow (i.e., irrigation) (Figure 5.5). All these constructs serve to wall the organism off from the surrounding sediment and should reduce the organism's direct exposure to interstitial water. Also, the open channel to the surface facilitates the ventilation of overlying water. Consequently, tubicolous species ventilate relatively more overlying water than free-burrowing species without siphons extending into the overlying water.

Besides reducing contact with interstitial water, the differential permeability of tubes/burrows changes the chemical composition of water in the tube compared to the surrounding interstitial water. Aller's studies on small inorganic solutes suggests that tubes act as "molecular sieves" (Figure 5.6).[30,31] The passage of certain anions (Br^-) through the tube/burrow walls were hindered relative to cations (NH_4^+) because of negative charges on the inside of the tube.

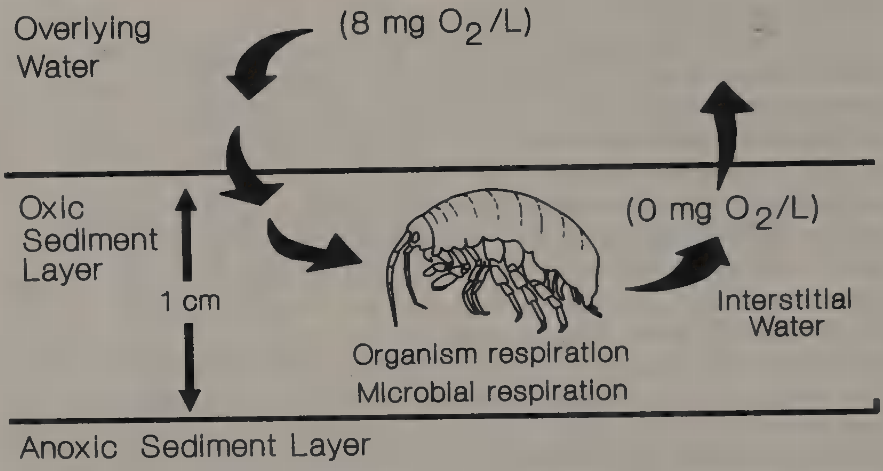

Figure 5.4. Calculation of turnovers of interstitial water required to maintain oxic sediment layer. Assuming equilibrium and no other source of oxygen input, the O_2 advected from the overlying water into the oxic sediment layer equals the O_2 respired by infauna and microbes (including chemical oxygen demand). Therefore, the number of turnovers of the interstitial water in the oxic layer can be estimated from the volume of overlying water required to supply the O_2 consumed by benthic respiration. It is assumed that all the O_2 consumption occurs in the oxic layer so areal measurements of benthic respiration ($g/m^2 \times$ day) can be expressed on basis of the volume of the oxic layer ($g/m^2 \times$ cm $\times$ day). Oxygen concentration in the overlying water is also expressed on a volume basis (g/m^2 cm). To calculate the volume of interstitial water, it is necessary to estimate the depth of the oxic layer and the percentage of the oxic layer consisting of interstitial water (i.e., porosity). The minimum number of turnovers required to meet the benthic oxygen demand can then be calculated from the oxygen concentration in the overlying water:

$$\text{Turnovers/day} = O_2 \text{ consumption}/(O_2 \text{ conc. OW} \times \%\text{IW}/100)$$

The turnovers can be calculated assuming the following:

- Depth of oxic layer = 1 cm.
- O_2 consumption rate = 3.38 g/(m^2 × cm × day). From Kemp and Boynton.[42]
- Interstitial water volume (%IW) = 50% of the oxic layer.
- O_2 conc. overlying water (OW) = 8 mg/L = 0.08 g/(m^2 × cm).
- 100% of the interstitial water is replaced each turnover.
- 100% of the oxygen is consumed each turnover.
- 100% of the oxygen consumption occurs in the oxic layer.

Using these values, a minimum of 84 turnovers of the interstitial water in the oxic layer are required to supply the respired oxygen. With this turnover rate, pollutants would need desorption rates on the order of hours to maintain equilibrium pollutant concentrations in the interstitial water.

Other solutes were restricted in their passage through the tubes due to consumptive reactions within the tube and by sorption on the tube.

Using Aller's work as a base, it is possible to postulate four different processes modifying the pollutant composition of tube/burrow water versus the

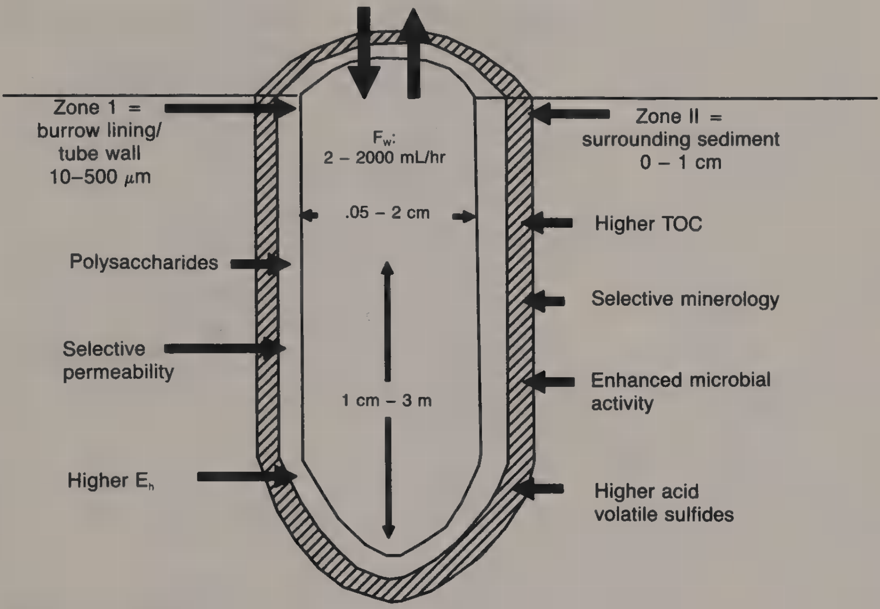
Zone 1 =
burrow lining/
tube wall
10–500 μm
F_w:
2 – 2000 mL/hr
Zone II =
surrounding sediment
0 – 1 cm
.05 – 2 cm
Higher TOC
Polysaccharides
Selective minerology
Selective
permeability
1 cm – 3 m
Enhanced microbial
activity
Higher E_h
Higher acid
volatile sulfides

Figure 5.5. Properties of infaunal tubes and burrows. A generalized schematic to illustrate the structure and the range in the dimensions and flux of water (F_W or "irrigation") of tubes and burrows. The actual dimensions and shapes vary greatly among species. The irrigation rates are from Lee and Swartz.[14] The other characteristics are from Aller and Yingst,[29] Aller,[30] and Aller et al.[31]

surrounding interstitial water (Figure 5.6). First, higher K_{ow} neutral organics would bind to the organic matter of the tubes/burrow, hindering their diffusion into and through the tube.

Second, passage of large molecules may be hindered in their passage through tubes by steric effects, while polar molecules may be hindered by charge interactions. Dissolved organic matter (DOM) consists of a high percentage of large molecules so DOM concentration may be reduced as interstitial water passes through a tube/burrow wall. The reduction in DOM may enhance the relative proportion of truly dissolved pollutant to DOM-bound pollutant, resulting in an increase in the bioavailability of organic pollutants (see Landrum et al.[32] and Servos and Muir[33] for effects of DOM).

The third process relates to the presence of higher levels of acid volatile sulfides (AVS) in certain burrow walls.[29] AVS may "bind" free metals and thus control their bioavailability, as suggested by the relationship between cadmium toxicity and AVS levels in sediments.[34] With the higher AVS concentrations, the concentrations of free metals may decline as they diffuse through the burrow wall, reducing their bioavailability.

The last process is the enhanced microbial activity in tube/burrow walls,[29] which could result in enhanced microbial degradation of organic pollutants. However, it is unclear whether the degradation would be sufficiently rapid to

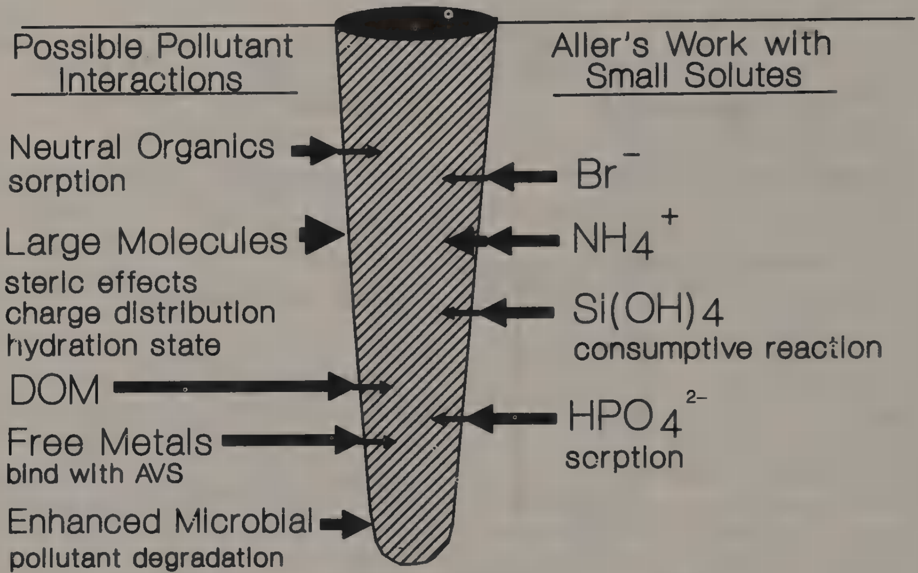
Possible Pollutant Interactions
Neutral Organics sorption
Large Molecules steric effects charge distribution hydration state
DOM
Free Metals bind with AVS
Enhanced Microbial pollutant degradation
Aller's Work with Small Solutes
Br⁻
NH4⁺
Si(OH)4 consumptive reaction
HPO4²⁻ sorption

Figure 5.6. Tubes/burrows as molecular sieves and possible effects on interstitial water pollutants. Tube/burrow walls acted as molecular sieves with small inorganic solutes.[30] Tube/burrow walls affected diffusion of solutes by charge interactions (slower diffusion of Br^- compared to NH_4^+), consumptive reactions within the tube ($Si(OH)_4$), and sorption to the tube/burrow (HPO_4^{2-}). Similar reactions can be postulated for pollutants. In particular, diffusion of neutral organics may be hindered by sorption to the TOC of the wall or by steric effects. Free metals may bind with the acid-free sulfides (AVS) present in high concentrations in certain burrow walls.[29] Organic pollutants may be degraded more rapidly as a result of the enhanced microbial activity in tube/burrow walls.

affect the exposure of the tube/burrow inhabitant or whether the degradation would result in a more toxic intermediate breakdown product.

TRANSFER PROCESSES

Once an organism is exposed, the transfer of the pollutant from the water or sediment into the organism is primarily regulated by two processes. The first is the ease of "release" or "extraction" of the pollutant from the aqueous or particulate phase, and the second is the assimilation of the released pollutant by the gill, gut, or integument. Bioavailability is reduced if either process is rate limited. In the following discussion, it is assumed that interstitial water pollutants are accumulated only at the gill surface, whereas pollutants sorbed to solids are accumulated only in the gut.

Extraction and Assimilation of Interstitial Water Pollutants

Truly dissolved pollutants do not have to be "extracted" from the water per se, so the important processes are those occurring at the gill membrane. However, there is growing evidence that a substantial percentage of the aqueous-phase organic pollutants are bound to DOM and that these bound pollutants have a reduced bioavailability.[32,33] The size of the freely dissolved pool in interstitial water is thought to be small,[5] suggesting that the rate of desorption from the DOM-bound pool is one of the factors regulating the availability of organic pollutants (Figure 5.7). To gain a better understanding of the role of interstitial water, future studies should attempt to separate the free and bound pools, though as of yet it is not clear which of the several techniques (dialysis, ultrafiltration, photolysis, hydrolysis, gas purging, and reverse-phase separation) is most suitable.

The major role organisms have on the extractability of interstitial pollutants is by influencing DOM concentrations. Infaunal organisms and microbes can increase the concentration of DOM through excretion or by in situ decomposition. For example, decomposing marine diatoms released humic and fulvic acids, though none were found in the living cells.[35] Infaunal organisms can also reduce DOM by directly assimilating dissolved carbohydrates and amino

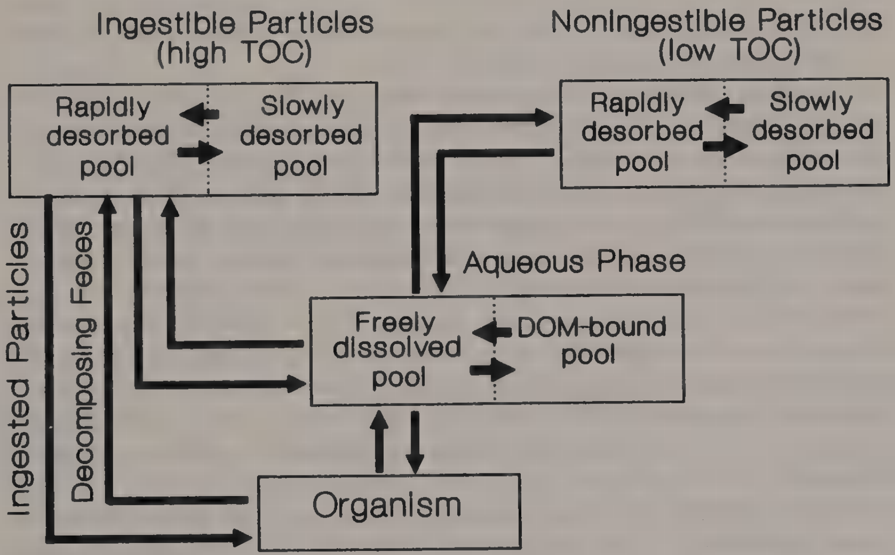
Ingestible Particles
(high TOC)
Noningestible Particles
(low TOC)
Rapidly desorbed pool
Slowly desorbed pool
Rapidly desorbed pool
Slowly desorbed pool
Ingested Particles
Decomposing Feces
Aqueous Phase
Freely dissolved pool
DOM-bound pool
Organism

Figure 5.7. Conceptual model of sediment bioavailability. Conceptual model showing the aqueous- and solid-phase pollutant pools in sediments and the important transfers among the pools and infaunal organisms. *Ingestible particles* are the smaller, high TOC particles ingested by selective deposit feeders. *Noningestible particles* are the larger, lower TOC particles rejected or consumed only in small amounts by selective deposit feeders. Diagram modified from Landrum.[5]

acids, though it seems unlikely that infaunal organisms could substantially reduce interstitial water DOM concentrations.

Once a dissolved pollutant comes in contact with the gill surface, uptake is regulated by the physical/chemical properties of the pollutant and the characteristics of the membrane. With fish, the efficiency of gill uptake (E_{PW}) of dissolved organic pollutants initially increased from a log K_{ow} of 1, then plateaued from log K_{ow} of 3 to 6, and declined at higher K_{ow}'s.[1,36] There have been no comparable multiple compound studies with infaunal invertebrates, but the similarity of the gill uptake efficiency of HCB for *Macoma*[28] to that reported for fish[1] suggests a qualitatively similar pattern may occur.

Extraction and Assimilation of Sorbed Pollutants

One route of uptake from pollutants sorbed to solids is desorption in the gut and then assimilation of the dissolved pollutant across the gut membrane. Sorbed pollutants can be divided into a "rapidly reversible pool" and a "slowly reversible pool."[37] Pollutants in the rapidly reversible pool desorb within minutes to hours, whereas pollutants in the slowly reversible pool take days or months to desorb. Because most deposit feeders have a gut retention time on the order of hours, uptake from desorbed pollutants should be primarily from the rapidly reversible pool. This assumes the digestive processes do not greatly increase the release of the slowly desorbed pool compared to laboratory techniques to measure desorption. The validity of this assumption needs to be tested because the gut is a harsh environment evolved to promote the assimilation of organic molecules.

Other than modifying gut retention time, organisms can potentially influence the desorption of pollutants in the gut through three mechanisms. The first is the stripping of the rapidly desorbed pool during digestion with a subsequent reduction in the bioavailability of the defecated particles. The importance of this process is suggested by the reduction in the K_s for phenanthrene measured in sequential sets of *Pontoporeia* exposed to the same sediment.[5] In the sediment exposed to three sets of amphipods over 6 weeks, the K_s declined by about 60% compared to about a 34% reduction in sediment aged without organisms. In benthic communities with rapid sediment reprocessing rates,[14] biological stripping of the rapidly desorbed pool may result in a general decrease in bioavailability. Consequently, bioavailability of sorbed pollutants may vary with the density and types of organism comprising a benthic community.

The second factor is how an organism's feeding method affects the "age" of ingested sediment. The size of the rapidly reversible pool decreases with equilibration time,[37] so recently polluted sediments should have a higher bioavailability than older sediments, which have had more time to equilibrate. Therefore, a species feeding on the surface would ingest sediment with a higher bioavailability than a deep-feeding species. The evidence for decreases in bioavailability over short aging periods includes the approximate 34% reduction in the bioavailability of phenanthrene after storing the spiked sediment an extra 4 weeks.[5] To examine the effects of aging over years or decades, it is worth reanalyzing a study in which we measured the accumulation factors (AF = lipid-normalized tissue residue/sediment pollutant concentration on organic carbon basis) for PCBs, DDE, DDD, and five PAHs using surface (0–2 cm) and deeper sediments (4–8 cm or 8–12 cm) collected at three sites off the Los Angeles County sewage discharge.[16] The AFs were measured in the laboratory by exposing *Macoma* to the sediments for 28 days. Because AFs are measures of steady-state body burdens rather than the rate of uptake, AFs are used only as a comparative measure of bioavailability. Although the ages of the sediment at different strata are unknown, these data can be reinterpreted assuming that the deeper sediments are several years to decades older.[15]

The ratio of the AFs derived from the surface sediment to the AFs from the deeper sediment averaged 2.4 for the 29 site-chemical combinations ($p < 0.05$, paired t-test), and the higher AF occurred in the surface sediment in 20 cases ($p < 0.05$, chi square test). The higher AFs in the surface sediments suggest the pollutants sorbed to the younger surface sediments were more bioavailable. The apparent increase in bioavailability in surface sediment was not related to pollutant type, though it was related to site, with the greatest enhancement at the reference site. Of the nine compounds at the reference site, the ratio of the surface AF to deep AF was > 2 in 8 cases. In comparison, this ratio was < 2 in all 20 comparisons at the two polluted sites. The cause for this apparent trend is not known.

The third process potentially affecting the uptake of sorbed pollutants is the increase in pollutant fugacity and pollutant concentration in the gut as the volume of food decreases and lipids are hydrolyzed during digestion.[23] Digestion alters the thermodynamic gradient such that there is a net flux of pollutant from the gut contents into the gut. This digestive-driven change in gut fugacity should have the greatest affect on the tissue residues of slowly eliminated or metabolized compounds (e.g., PCBs). This process may explain why tissue residues of high K_{ow} compounds often exceed the concentrations predicted from thermodynamic partitioning with the external environment (e.g., Connolly and Pederson[38]).

Another process that could account for tissue residues in excess of that predicted by simple partitioning is facilitated or active uptake in which the organism expends energy to incorporate [illegible] compound. For example, ion pumps can carry charged solutes against a thermodynamic gradient. Phagocytosis, in which digestive cells engulf organic particles, could transport neutral organic pollutants sorbed to the engulfed particle into the gut membrane regardless of the thermodynamic gradient. Although these processes are known to occur, their exact role in the uptake of metals is unclear.[39] Based on our studies with the PCB congeners, their role in the uptake of neutral organics is also unclear. As discussed below, there was no apparent gut uptake of decachlorobiphenyl by *Macoma*. One interpretation is that the active uptake of neutral organics was trivial and lost in the noise. Alternatively, active uptake may have carried the decachlorobiphenyl into the gut membrane, but then the PCB congener was trapped in the membrane by steric or other effects. If the latter interpretation is true, then active uptake could still be an important uptake mechanism for compounds not subject to steric effects.

Understanding the importance of these gut processes and the magnitude of uptake from ingested sediment requires estimates of the efficiency of uptake from ingested sediment (E_{PSi}). To date, there are only two published measurements of the assimilation of organic pollutants from ingested sediment—a value of 24% for a hexachlorobiphenyl with oligochaetes by Klump et al.[40] and a value of 39–56% for HCB with *Macoma*.[20] One problem with the dual label approach used by Klump is the possibility of underestimating the efficiency of uptake if the organism selects for high TOC particles, as was discussed above. To avoid this problem, we derived a direct measurement approach,[20] using a "clambox" (see Specht and Lee[41]) that corrects for sediment selection by using TOC as a pollutant tracer.

Besides the measurement with HCB, we have measured E_{PSi} for 13 PCB congeners and HCB (work in progress). Based on the preliminary data, there is an indication that E_{PSi} declines with K_{ow}. Sediment uptake efficiencies declined from about 90% for 2,2′,5-trichlorobiphenyl (IUPAC #18) to 0% for decachlorobiphenyl (IUPAC #209) over a log K_{ow} range of 5.2 to 8.2. The small amount of accumulation of the decachlorobiphenyl over 10 days (Figure 5.2) is thought to be due to sorption to the integument and/or the gut wall rather than internal tissues. Qualitatively similar declines in efficiency at high K_{ow} have been reported in fish for both uptake of dissolved pollutants across the gills (E_{PW})[1] and for the uptake efficiencies of food in the gut (E_{PF}).[23] However, in these studies the decline in the efficiency terms began at a higher log K_{ow}: $>$ 6.0 for gill uptake and $>$ 6.5 for gut uptake.

CONCLUSIONS

The first purpose of this chapter was to propose K_s, the uptake rate coefficient, as the definition for sediment bioavailability. Standardization of terminology will help avoid confusion and assist in making quantitative comparisons among studies. Other measures, such as steady-state tissue residues, can generate insights into the processes regulating bioavailability but, as defined here, are not measures of sediment bioavailability per se. The second purpose was to identify potential processes by which organisms can alter their exposure regime and the efficiency of pollutant assimilation. Not all the processes discussed will be important for any single species, sediment type, or pollutant, and future studies may well demonstrate that some of the processes have a trivial effect on sediment bioavailability. However, there is sufficient data to warn us that, at least in some cases, standard sampling and chemical procedures can do a poor job of estimating an organism's exposure. The documentation of these biological processes should also warn of the danger of treating infaunal organisms as static players driven solely by thermodynamic partitioning with the external environment (see Figure 5.7).

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CHAPTER 6

The Influence of Water Column Dissolved Organic Carbon on the Uptake of 2,2′,4,4′,5,5′-Hexachlorobiphenyl (PCB 153) by *Daphnia magna*

Hayla E. Evans

INTRODUCTION

In recent years, there have been a plethora of laboratory studies that have demonstrated that the bioavailability of many organic contaminants can be changed by the presence of humic acid (HA). For example, it has been shown that HA can reduce the uptake of dioxin, polychlorinated biphenyls (PCBs), and/or certain polycyclic aromatic hydrocarbons (PAH) by pelagic invertebrates such as *Daphnia magna*;[1,2] by benthic invertebrates such as *Pontoporeia hoyi*[3] and *Crangonyx laurentianus*;[4] and by fish such as *Lepomis macrochirus*,[5,6] *Salmo gairdneri*,[7,8] and *S. salar*.[9,10]

Similarly, the dissolved organic carbon (DOC) extracted from sediment interstitial waters has been shown to decrease the uptake rate constant of two PAHs (pyrene and benzo(a)pyrene) and a PCB (2,2′,4,4′-tetrachlorobiphenyl) by *P. hoyi*.[11] On the other hand, HA had no effect on *Daphnia* accumulation of several other PAHs, including anthracene, dibenzanthracene, dimethylbenzanthrazene, and naphthalene.[1,2] Naphthalene accumulation by sunfish was also unaffected by the presence of HA,[5] as was anthracene accumulation.[6] However, HA actually increased the accumulation of methylcholanthrene by *Daphnia*,[1] although these results were contradictory to those obtained later by McCarthy et al.[2]

Fewer bioavailability investigations have been carried out using "natural" or water column DOC, presumably because of the difficulty in obtaining a wide range of DOC concentrations in situ. This is unfortunate because it is becoming increasingly apparent that water column DOC may be considerably less effective than either HA or sediment interstitial DOC in the binding of hydrophobic organic contaminants in lakes.[12–15] Furthermore, in laboratory investigations in which invertebrates are used, the effect of lipid content (or condi-

Table 6.1. Chemical Composition of the Water Used for PCB 153 Uptake Experiments by *D. Magna*

	Composition (mg/L)							pH	Cond (μmhos/cm)	Color (Hazen Units)
	Ca	Mg	Na	K	Cl	SO_4	DOC			
SLW	2.50	0.65	0.90	0.56	0.74	3.15	<0.1	—	—	—
FLW										
4/11/89	2.45	0.63	1.03	0.70	1.24	6.9	7.7	—	36	69
5/9/89	2.75	0.66	1.31	0.52	1.50	6.6	7.5	—	33	70
6/14/89	2.90	0.62	1.49	0.83	1.51	6.4	8.7	—	—	—
1978–86[a]	2.64	0.62	0.84	0.55	0.74	7.2	8.5	5.6	29	110
2X-FLW										
+6/14/89	5.95	1.32	2.50	1.19	2.58	12.05	12.7	—	—	—

Note: SLW = simulated lake water, FLW = filtered Fawn lake water, 2X-FLW = concentrated filtered Fawn lake water (see text for explanation).

[a] 1978–1986 whole-lake "ice-free" mean, n = 8 (from Reid and Girard).[32]

tion) of the experimental animals on contaminant uptake has been largely ignored. This practice also may be unwise because studies have shown that lipid content contributes to experimental variability[16] and to the ultimate concentration of pollutants such as PCBs in zooplankton.[17] Certainly for fish, it is widely accepted that the bioconcentration of hydrophobic organic contaminants is largely dependent on the animals' lipid fraction.[18,19]

Therefore, the purpose of this study was to investigate the uptake of 2,2′,4,4′,5,5′-hexachlorobiphenyl (PCB 153) by the freshwater cladoceran *Daphnia magna* using water column DOC at various concentrations. Also an attempt was made to examine and maintain a consistent lipid content in the animals.

MATERIALS AND METHODS

Collection and Preparation of the Water

Water for the uptake experiments was either DOC-free simulated lake water (SLW) or filtered lake water (FLW) collected from Fawn Lake, a small (0.858 km²) brown-water lake located ~250 km north of Toronto, Ontario. The SLW was prepared by adding 7 mg/L $MgSO_4 \cdot 7H_2O$, 2.5 mg/L Na_2SO_4, 1 mg/L KCl, 0.5 mg/L $CaCl_2 \cdot 2H_2O$, and [illegible] mg/L $CaCO_3$ into distilled deionized water. The resulting chemical composition of the water (Table 6.1) is typical of soft-water lakes in south central Ontario.

Water was collected from the outflow of Fawn Lake in 4-L amber glass bottles on April 11, May 9, and June 14, 1989. Within 24 hr, the water was pumped through a 0.45-μm Gelman flow-through filtration system. The filtered water was refrigerated, in darkness, and used for the uptake experiments within 2 weeks of collection. The concentrations of Ca, Mg, Na, K, SO_4, Cl, and DOC in the lake on each sampling day, together with long-term data for

Fawn Lake are given in Table 6.1. All analyses were conducted by the Ontario Ministry of the Environment (OMOE).[20]

The FLW from the April 11th collection was not used directly for uptake experiments. Rather, it was evaporated at 50°C in a drying oven in order to reduce the volume of water by ~130 times. This concentrate was then added back into FLW that had been collected from the lake on June 14th, so that an increase in the ambient DOC concentration of the lake water could be achieved. The resulting ionic composition of this water (i.e., 2X-FLW) is given in Table 6.1. Thus, the DOC concentrations of the water used in the uptake experiments were 0, 7.5, and 12.7 mg C/L.

Culturing of Animals

Daphnia magna were obtained from a culture maintained at the University of Toronto; however, for these uptake experiments, a new culture was started by placing a single daphnid into SLW. The second (i.e., F_2) generation of offspring from this *Daphnia* were then distributed between two 4-L jars (one containing SLW and one containing autoclaved FLW) and fed ~1 mL of a *Chlamydomonas* culture daily. *Daphnia* for the uptake experiments were taken either from the jar containing SLW (for the SLW experiments) or the jar containing autoclaved FLW (for the experiments using FLW and 2X-FLW). SLW or autoclaved FLW was added to each jar as required.

Experimental Design

To begin each experiment, the amber glass bottles containing 4 L of SLW, FLW, or 2X-FLW were spiked with ~4 μL of ^{14}C-labeled PCB 153 (~4 μg PCB/L; specific activity = 9.4 μCi/μmol; Pathfinder Laboratories, St. Louis, MO) in hexane carrier. The bottles were capped and allowed to equilibrate for 17–70 hr. After equilibration, a 2-mL subsample of water was taken for the determination of total PCB concentration (i.e., C_T in dpm/mL). At the same time, the amount of PCB bound to the DOC (i.e., C_B in dpm/mL) was ascertained using Sep-Pak C_{18} cartridges (see Evans[14]). Briefly, a 5-mL glass syringe was used to rinse 2.5 mL of water through the column at a flow rate of ~10 mL/min, following which an additional 2.5 mL of water was passed through the column. A 2-mL sample of the eluant was then obtained and immediately placed into a glass vial containing 18 mL of aqueous counting scintillant (ACS, Amersham).

Next, 199 ± 1 mL of the ^{14}C-PCB 153 labeled water was weighed directly into twenty 250-mL Erlenmeyer flasks. Prior to the actual start of the experiment, *Daphnia* were examined microscopically and hand-selected to be of approximately similar size and lipid content (following the lipid index of Tessier and Goulden[21]). At time zero, a single *Daphnia* was added to each flask.

Five replicate flasks were sampled at 1-, 2-, 4-, and 8-hr time intervals. Total PCB concentration (C_T) was determined on 2-mL samples taken from each flask. The individual *Daphnia* were then removed from the water, put into 1–3 mL of clean water, blotted, and weighed immediately to determine wet weight. Each daphnid was then placed directly into a glass vial containing ACS.

Previous experiments that I had conducted had shown about a 5% decrease in the wet weight of a daphnid within the first minute after blotting. Consequently, the time between the blotting and weighing of the animals was kept as short and as constant as possible in order to minimize variation in *Daphnia* wet weights. For the 8-hr samples only, the *Daphnia* were quickly reexamined (prior to being weighed), and the lipid index was ascertained. In addition, the amount of PCB bound to DOC (C_B) was determined (as explained above) on a centrifuged (1000 rpm for a minimum of 10 min) subsample of water taken from one of the flasks.

In total, twelve uptake experiments were performed: five with the SLW ([DOC] = 0 mg/L), four with the FLW ([DOC] = 7.5 mg/L), and three with the 2X-FLW ([DOC] = 12.7 mg/L). To account for the extent of accumulation attributed to sorption to the carapace as well as to that occurring through passive diffusion, an uptake experiment was also conducted using dead *Daphnia* in SLW. The same procedure was applied except that the animals were heat-killed (50°C for 10 min) prior to the experiment.

Depuration of radiolabeled PCB by the *Daphnia* was measured in SLW. As with the uptake experiments, 4 L of water were spiked with ^{14}C-labeled PCB and allowed to equilibrate for 47 hr. Then the water was weighed into twenty Erlenmeyer flasks, and one *Daphnia* (hand-selected as before) was added to each flask. After a 4-hr exposure to the PCB, the *Daphnia* and the water in five of the flasks were sampled. Meanwhile, the remaining fifteen *Daphnia* were transferred to fifteen flasks, each containing 199 ± 1 mL of clean, nonradiolabeled SLW. These flasks were then sampled after 2, 4, and 8 hr (following the procedures outlined above).

All the samples (i.e., water and *Daphnia*) were counted for 10 or 20 min on a Beckman LS 7000 liquid scintillation counter. In order to calculate dpm, the samples were corrected for counting efficiency (92.5–95.5%) and background (44–55 dpm) using the H number method of quench monitoring (an external standardization technique).

Calculations

After exposure to a known concentration of contaminant in water, the uptake rate constant (K_u in mL/mg wet weight/hr) for PCB 153 by *Daphnia* can be determined from the conventional uptake-depuration equation for aquatic biota:

$$dC_D/dt = K_uC_T - K_dC_D \tag{6.1}$$

where C_D = concentration of PCB in the *Daphnia* (dpm/mg)
C_T = total concentration of PCB in the water (dpm/mL)

K_d = depuration rate constant (hr^{-1})
t = time (hr)

In these experiments, the total concentration of PCB 153 in the water changed approximately linearly through time according to the equation:

$$C_T = C_{T(0)}(1 + K_w t) \tag{6.2}$$

where $C_{T(0)}$ = predicted concentration of PCB in the water at time zero (dpm/mL)
K_w = rate of loss/increase of PCB (hr^{-1}) in the water

Thus, for each uptake experiment, the data on the change in PCB concentration in the water through time were fitted to Equation 6.2, and the values for $C_{T(0)}$ and K_w were ascertained. Substituting Equation 6.2 into Equation 6.1 yields

$$dC_D/dt = K_u C_{T(0)}(1 + K_w t) - K_d C_D \tag{6.3}$$

Equation 6.3 can be integrated using an integrating factor method. The result is, for $C_D = 0$ when $t = 0$,

$$C_D = (K_u/K_d)C_{T(0)}[(1 - e^{-K_d t}) + (K_w/K_d)(K_d t - 1 + e^{-K_d t})] \tag{6.4}$$

Depuration of PCB 153 (K_d) was determined by placing contaminated *Daphnia* into clean water as described above. From Equation 6.1, the concentration of PCB 153 in the animals through time is given by

$$dC_D/dt = -K_d C_D \tag{6.5}$$

which yields after integration

$$C_D = C_{D(0)} e^{-K_d t} \tag{6.6}$$

or

$$\ln C_D = \ln C_{D(0)} - K_d t \tag{6.7}$$

where $C_{D(0)}$ (dpm/mg) is the initial (i.e., contaminated) concentration of PCB 153 in the *Daphnia*. Consequently, a semilog plot of C_D versus time should give a straight line with slope = K_d. The K_d value, together with the values for K_w and $C_{T(0)}$ determined from Equation 6.2 and the C_D measurements made at each sampling period, were substituted into Equation 6.4, so that four estimates of the uptake rate constant, K_u, could be made (i.e., at 1, 2, 4, and 8 hr). The average of these estimates was used to test for differences in the K_u values among the various DOC concentrations using a one-way analysis of variance (ANOVA).

The association coefficient calculated on the basis of DOC (K_{DOC} in mL/g C) can be determined from

$$K_{DOC} = \frac{C_B/[DOC]}{C_T - C_B} \tag{6.8}$$

where [DOC] is the DOC concentration in the water in g/L. The fraction of hydrophobic contaminant that passes through the Sep-Pak column in the absence of DOC was previously determined to be 5% for PCB 153,[14] and so a breakthrough factor (BF = 0.05 C_T, in dpm/mL) was subtracted from C_B prior to calculating K_{DOC} values.

RESULTS AND DISCUSSION

After equilibration of the water with the PCB, that is, at the beginning of each uptake experiment, and also at the 8-hr sampling interval, the amount of PCB bound to DOC was less than 5 dpm/mL in the FLW experiments (i.e., < 9% of the PCB 153 was bound to the DOC), and less than 14 dpm/mL in the 2X-FLW experiments (i.e., < 13% of the PCB was bound to the DOC). Since counting errors at these low levels are very high (i.e., $\pm\sqrt{N}/N \times 100\%$, where N is the total number of counts), K_{DOC} values were not determined in any of the FLW experiments nor were they determined at the beginning of the 2X-FLW experiments. However, at the 8-hr sampling period, the association coefficient ranged between 8.2×10^3 and 1.1×10^4 mL/g C. These K_{DOC} values are about one-third to one-half of the value of 2.6×10^4 mL/g C reported for the association of PCB 153 with the DOC in Lake Michigan ([DOC] = 0.83–5.8 mg C/L)[22] and also about one-third to one-half of the values previously reported for Fawn Lake.[14]

While the slightly lower K_{DOC} values calculated in this study are due most likely to natural variation, alternately some PCB could be associating with hexane carrier still remaining in the 4-L bottles after the equilibration period. This "hexane-bound" PCB would not pass through the Sep-Pak column and would result in an anomalously low K_{DOC} value. A change in the chemistry, and thus the binding capacity, of the water during the evaporation and subsequent dilution procedure is also a possibility, although a similar concentration procedure previously was found to have no effect on the molecular weight distribution of the DOC, < 5000 daltons in size, collected from this lake.[23]

The recovery (i.e., the total amount of isotope in the water + *Daphnia* at the end of the experiment divided by the total amount of isotope added at the start of the experiment) of PCB 153 measured in one SLW experiment was found to be 60% after the 8-hr uptake experiment (following 18-hr equilibration). This is comparable to the recovery obtained during other investigations where no *Daphnia* were present.[14] About 94% of the recovered isotope was in

Table 6.2. Linear Regressions of the Total PCB Concentration in the Water (C_T,dpm/mL) versus Time (hours) in each of the 12 Uptake Experiments

Experiment		p	K_w
4400–SLW	C_T = 89.2 – 2.58 (time)	0.086	–0.029
4700–SLW	C_T = 75.9 – 1.45 (time)	0.238	–0.019
4300–SLW	C_T = 75.7 – 2.86 (time)	0.001	–0.038
4200–SLW	C_T = 65.9 – 2.77 (time)	0.088	–0.042
4100–SLW	C_T = 59.3 – 2.26 (time)	0.004	–0.038
3800–FLW	C_T = 57.0 – 1.03 (time)	0.160	–0.018
3500–FLW	C_T = 15.5 + 0.357 (time)	0.035	0.023
3900–FLW	C_T = 86.3 – 1.99 (time)	0.000	–0.023
4000–FLW	C_T = 92.9 – 1.60 (time)	0.080	–0.017
5100–2X–FLW	C_T = 184 – 6.83 (time)	0.044	–0.037
4900–2X–FLW	C_T = 82.9 – 2.73 (time)	0.000	–0.033
5000–2X–FLW	C_T = 115 – 2.22 (time)	0.022	–0.019

Note: K_w = rate of loss/increase of PCB from the water (hr^{-1}) calculated according to Equation 6.2.

the water and 6% in the *Daphnia*. The 40% loss of isotope may have occurred as a result of coevaporation (with the hexane) during the equilibration period or when the radiolabeled water was being poured from the 4-L amber glass bottle into the Erlenmeyer flasks. Alternately, adsorption of the isotope onto the walls of the glassware may have occurred, especially in the absence of DOC.[24]

The results of the twelve linear regressions of the total PCB concentration in the water versus time according to Equation 6.2 are given in Table 6.2. In six of the twelve uptake experiments, there is a significant decrease in C_T through time ($p < 0.05$), and in one experiment, there is a significant increase in C_T through time. The corresponding values for K_w range between –0.019 and –0.042/hr in eleven of the uptake experiments, and 0.023/hr in one experiment.

The results of the depuration experiment are shown in Figure 6.1. In this figure, two data points (one at $t = 0$ and one at $t = 8$ hr), which were greater than three standard deviations from the mean of the other four points, were eliminated from the analysis. However, because biological variability is still large and elimination is very slow, the slope (K_d) of the semilog plot of C_D versus time is not significantly different from zero over the 8-hr depuration period ($p > 0.05$). Nonetheless, the K_d value of 0.0235/hr derived from these data was used as the best estimate of the depuration rate constant. Uptake of PCB 153 by direct sorption to the carapace of the *Daphnia* and by passive diffusion (ascertained using heat-killed *Daphnia*) was negligible (< 1 dpm/mg wet weight/hr, which was < 4% of the total PCB activity in the water).

Typical plots of C_D versus time are shown in Figure 6.2. It can be seen that generally C_D increases linearly through time, although in Figure 6.2c (5100–2X-FLW), the PCB concentrations in the *Daphnia* appear to plateau. This phenomenon was observed in other experiments in which there was a

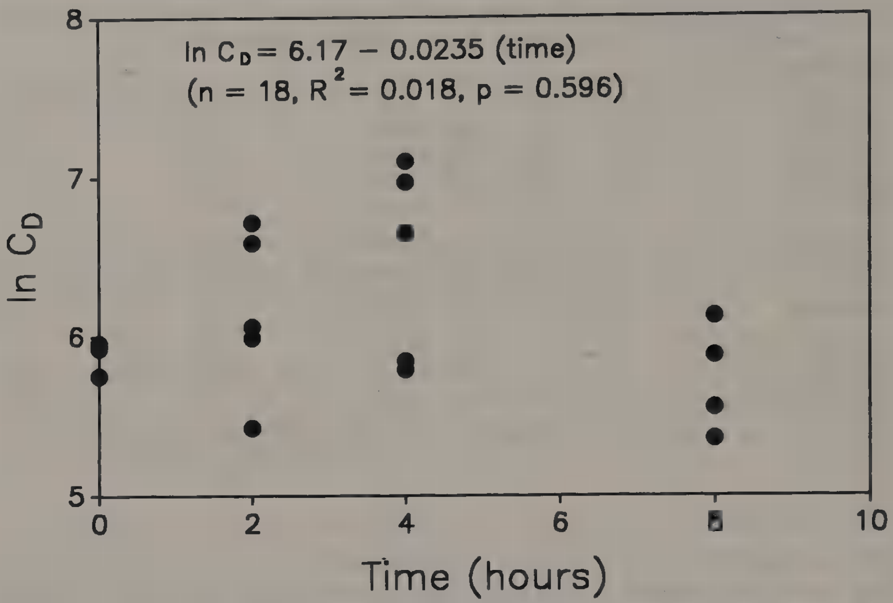

Figure 6.1 Depuration of PCB 153 by *Daphnia magna* following 4-hr exposure to the contaminant. C_D = concentration of PCB 153 in the *Daphnia* in dpm/mg wet weight.

significant decrease in C_T through time (see Table 6.2). Thus, the observed plateau in the data most likely is a reflection of decreasing water concentrations and not of steady state. An estimate of the amount of time required to reach steady state can be made from the depuration rate constant, K_d, since the half-life ($t_{1/2}$, in hours) of the PCB 153 in the *Daphnia* is given by

$$t_{1/2} = 0.693/K_d \tag{6.9}$$

Assuming a K_d value of 0.0235/hr (Figure 6.1), the half-life of PCB 153 in the *Daphnia* is calculated to be about 29 hr. Since the uptake experiments lasted only 8 hr, it is unlikely that steady state was attained. The observation that the plots of C_D continue to increase linearly over the 8-hr experiment (see Figure 6.2), except where there are changes in C_T, would also support this conclusion.

The wet weights of the *Daphnia* used for each experiment are given in Table 6.3. While they vary about threefold within each experiment (average coefficient of variation in wet weights = 26.8% ± 5.9 for the 12 uptake experiments, n = 20 for each experiment), an ANOVA revealed that the average wet weight of the *Daphnia* was not significantly different among the three DOC concentrations (p = 0.756) with average values of 0.950 ± 0.194 (n = 5), 1.049 ± 0.180 (n = 4), and 0.993 ± 0.212 (n = 3) mg wet weight for the SLW, FLW, and 2X-FLW experiments, respectively.

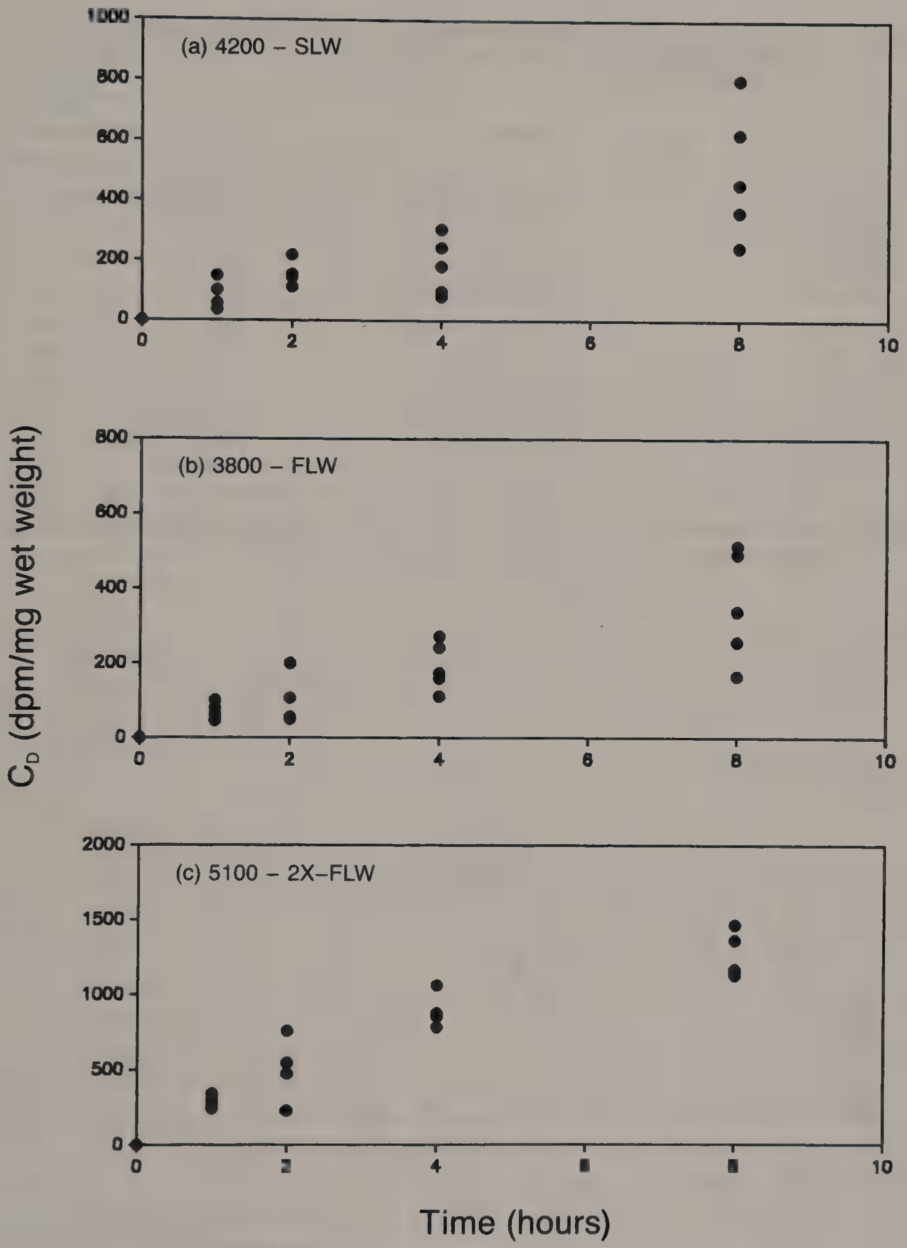

Figure 6.2 Uptake of PCB 153 by *Daphnia magna* through time in *(a)* SLW, *(b)* FLW, and *(c)* 2X-FLW. C_D = concentration of PCB 153 in the *Daphnia* in dpm/mg wet weight.

Table 6.3. DOC Concentrations, Wet Weights, and Lipid Indices of the *Daphnia*, and Average Uptake Rate Constants and Bioconcentration Factors for Each of the 12 Uptake Experiments

Experiment	[DOC] (mg C/L)	Wet Weight (mg)	Lipid Index	K_u (mL/ mg ww/hr)	BCF (mL/ mg ww)
4400–SLW	0	0.855 (0.298)	0.5	2.32 (0.94)	99
4700–SLW	0	0.682 (0.106)	0.5	1.81 (0.76)	77
4300–SLW	0	1.069 (0.259)	1.0	1.41 (1.17)	60
4200–SLW	0	1.184 (0.300)	1.5	1.16 (0.25)	49
4100–SLW	0	0.961 (0.332)	2.0	1.95 (1.73)	83
3800–FLW	7.5	1.293 (0.334)	1.0	1.06 (0.17)	45
3500–FLW	7.5	1.042 (0.261)	1.5	1.16 (0.30)	49
3900–FLW	7.5	0.995 (0.995)	2.0	1.56 (0.82)	66
4000–FLW	7.5	0.869 (0.869)	2.0	2.83 (0.85)	120
5100–2X–FLW	12.7	1.055 (0.280)	0.5	1.40 (0.22)	60
4900–2X–FLW	12.7	0.765 (0.211)	1.0	1.92 (0.46)	82
5000–2X–FLW	12.7	1.178 (0.414)	1.5	1.91 (0.33)	81

Notes: Average uptake rate constants (K_u) calculated from Equation 6.4. BCF calculated as K_u/K_d. Numbers in parentheses are one standard deviation.

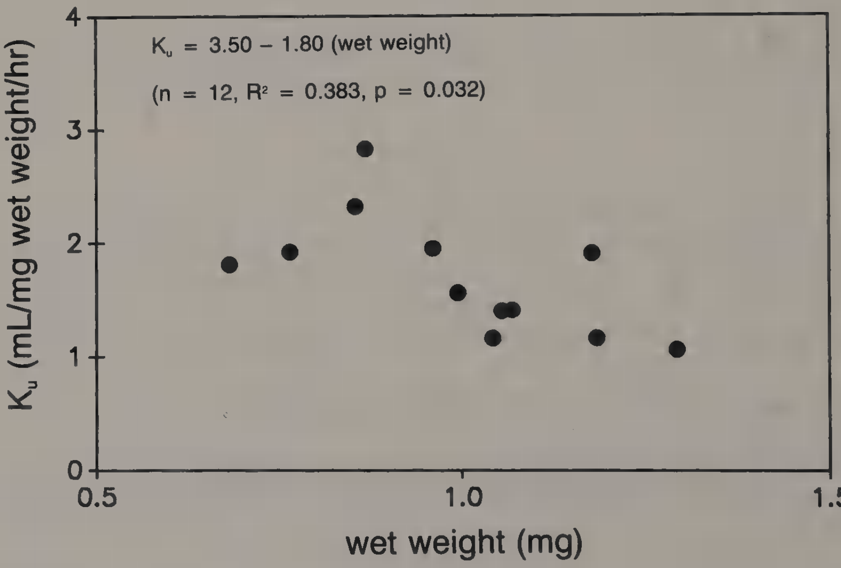

Figure 6.3 Relationship between the uptake rate constant, K_u (mL/mg wet weight/hr), for PCB 153 and the wet weight of the *Daphnia* (mg wet weight).

The average uptake rate constants, calculated according to Equation 6.4 are also given in Table 6.3. They range between 1.06 and 2.83 mL/mg wet weight/hr (mean = 1.71 ± 0.52, n = 12) which is comparable to, but slightly higher than, the value of 0.750 mL/mg wet weight/hr estimated by McCarthy et al. for the uptake of benzo(a)pyrene (BAP, log K_{ow} = 6.5) by *Daphnia* in water containing no HA.[2] It should be noted that these are conditional uptake rate constants because they are based on the total amount of PCB in the water, and not just the freely dissolved concentration. However, as discussed above, the results from the Sep-Pak experiments indicated that in all experiments only a small (and often indeterminate) fraction of the PCB was bound to the DOC.

There is a significant relation between the uptake rate constant and the *Daphnia* wet weights (n = 12, p = 0.032), as shown in Figure 6.3, with smaller *Daphnia* having slightly higher uptake rate constants than larger *Daphnia*. These results are similar to those reported by Landrum, who found that there was a significant increase in K_u values for tetrachlorobiphenyl and BAP as the weight of a benthic invertebrate, *Pontoporeia hoyi,* decreased.[25] Evans and Landrum report K_u values of 0.0535 and 0.0575 mL/mg wet weight/hr for PCB 153 by *Pontoporeia hoyi* and *Mysis relicta*, respectively.[26] While these invertebrates cannot necessarily be compared to *Daphnia*, my average K_u value of 1.71 mL/mg wet weight/hr is more than an order of magnitude higher than their values. However, the average wet weights of *Pontoporeia* and *Mysis* are 8 and 40 times greater than that of *Daphnia* (8.3 and 43 mg, respectively,[26] versus 1 mg for *Daphnia*). Thus, much of the difference between their results and my own could be attributed to the size of the animals rather than their habitat and physiology.

In order to test whether water column DOC has an effect on the uptake of PCB 153 by *D. magna*, a one-way ANOVA was conducted on the data. Since there were no significant differences in the average size of the animals among the three DOC concentrations, a direct comparison could be made between [DOC] and K_u values. The results showed no significant differences among the 3 DOC concentrations (p = 0.973), with average K_u values of 1.73 ± 0.446 (n = 5), 1.65 ± 0.814 (n = 4), and 1.74 ± 0.297 (n = 3) mL/mg wet weight/hr for the SLW, FLW, and 2X-FLW experiments, respectively (Table 6.3). These results assume that K_d is constant and equal to 0.0235/hr for each of the three DOC concentrations. While this assumption may result in a consistent bias in the calculated K_u values, this bias is small. For example, a threefold increase in the K_d value used in Equation 6.4 resulted in less than a 10% increase in the average K_u values calculated.

If the system has reached steady state, the bioconcentration factor (BCF, in mL/mg wet weight) can be measured directly as C_D/C_T. Alternately, it can be determined from K_u/K_d.[26] The BCFs calculated in this manner (Table 6.3) range between 45 and 120 mL/mg wet weight, which corresponds to a dimensionless BCF of $4.5–12 \times 10^4$ if the wet density of the *Daphnia* is assumed to be 1 g/mL. This BCF is very close to the value of 4.4×10^4 reported by Oliver and Niimi for the in situ bioconcentration of PCB 153 by all plankton in Lake Ontario (see their Table 1).[19]

An estimate of BCF can also be made from the empirical relationship developed by Mackay:[18]

$$\mathrm{BCF} = 0.048\ \mathrm{K_{ow}} \tag{6.10}$$

where K_{ow} is the octanol-water partition coefficient and the constant, 0.048, reflects the lipid content of the biota (i.e., 4.8% for fish). Data pertaining to the lipid content of pelagic zooplankton are scarce, but Wainman and Lean report seasonal values of about 0.8–1.5% (converted to a wet weight basis) for all zooplankton, including *Daphnia* spp., collected from Anstruther Lake, Ontario.[27] MacDonald and Metcalfe measured slightly lower lipid contents of 1.17, 0.17, and 0.22% for the zooplankton in Lakes Clear, Rice, and Scugog (Ontario), respectively.[28] Assuming a lipid content of 0.2–1.5% and a log K_{ow} value for PCB 153 of 6.9,[29] the predicted BCF from Equation 6.10 would be $1.6\text{–}11.9 \times 10^4$, which is very close to the measured values of $4.5\text{–}12 \times 10^4$. These results would therefore support the assumption that the dominant concentrating phase in *Daphnia*, as in fish, is lipid, and as a result, bioconcentration occurs via direct partitioning into the lipid fraction.

While lipid levels were ascertained visually in these experiments (see Table 6.3), rigorous statistical analysis of the data was not possible because the lipid index was not calibrated against "true" lipid concentrations. Nonetheless, there does not appear to be a trend in K_u in BCF with increasing lipid level. These results are similar to those reported by Landrum, who found no correlation between lipid content and the uptake rates of BAP and phenanthracene by *P. hoyi*.[25] On the other hand, the variation in the measured K_u values, at a given DOC concentration (Table 6.3), might be a result of the condition (i.e., lipid content) of the *Daphnia*, as has been found by others.[16] Since the lipid index of the *Daphnia* often dropped by 1 unit by the end of the 8-hr experiment, this factor should be considered in all studies involving invertebrates and other biota.

The results published in the literature for experiments conducted on invertebrates using "natural" (i.e., water column) DOC are somewhat mixed. Leversee et al. reported that the removal of DOC from two filtered natural waters resulted in about a 30% increase in the BCF of BAP by *Daphnia*.[1] Similarly, Kukkonen and Oikari[30] and Kukkonen et al.[31] found that the accumulation of dehydroabietic acid and BAP by *Daphnia* were distinctly reduced in natural humic waters, although no reduction in the accumulation of pentachlorophenol was observed.[30] Furthermore, Kukkonen et al. were unable to reconcile the decrease in the bioavailability of BAP with the fraction of the PAH that was bound to the DOC, suggesting that the BAP was, in fact, still available for uptake by the *Daphnia*.[31] Servos and Muir showed that epilimnetic DOC decreased the uptake of dioxin by the amphipod *Crangonyx laurentianus*, but their data suggest that epilimnetic (water column) DOC was less effective in reducing uptake than either HA or interstitial DOC.[4] In fact, Servos et al. found that water column DOC had little effect on the apparent uptake rate of dioxin by rainbow trout (*S. gairdneri*).[8]

In Fawn Lake, it is likely that the water column DOC has a much lower sorptive capacity for PCB than does HA and interstitial DOC and/or that the PCB 153 bound to the DOC is still available for uptake by the *Daphnia*. In these experiments, probably the former is true because given a K_{DOC} value of $\sim 10^4$ mL/g C and a [DOC] of $\sim 10^1$ mg/L, there is still about 90% of the PCB 153 available for uptake by the *Daphnia*. The results from the present study therefore suggest that water column DOC in lakes may be having a negligible effect on the cycling of PCB 153 and perhaps other hydrophobic organic contaminants. Furthermore, both the size (wet weight) and condition (lipid content) of the *Daphnia* are important factors affecting both the magnitude of, and the variability in, the measured BCFs and uptake rate constants. Thus, these variables should be considered in all bioavailability studies.

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CHAPTER 7

Binding and Bioavailability of Organic Micropollutants in Natural Waters: Effects of the Quality and the Quantity of Dissolved Organic Material

Jussi Kukkonen, John F. McCarthy, and Aimo Oikari

INTRODUCTION

The dissolved organic material (DOM) pool in natural waters consists of a variety of organic molecules. While some of these molecules have a defined chemical structure, most of the organic material in natural waters has no readily identifiable structure, and the members of this heterogeneous group of organic macromolecules are referred to as *humic substances*. DOM is an important factor in water chemistry and aquatic toxicology because a number of studies have demonstrated that it can bind both metals[1] and hydrophobic organic pollutants.[2–6] The magnitude of the binding, expressed as a partition coefficient, K_p, is related to the hydrophobicity of the contaminant.[5,7] However, the affinity of the organic matter for binding a given contaminant appears to vary among waters from different sources.[3,8,9] The underlying causes of the observed variability in binding affinity of different waters for organic contaminants is not fully understood and hampers attempts to describe and predict the importance of natural organic matter in the transport and fate of organic pollutants in aquatic systems.

The bioavailability of metals and organic pollutants is also affected by dissolved organic matter. DOM reduces the bioavailability of pollutants, and the magnitude of the decrease is related to the extent of the binding between the contaminant and the organic matter.[9–12] Thus, the capability to predict the role of organic macromolecules on the accumulation and toxicity of hydrophobic organic contaminants in aquatic environments is dependent on, and limited by, the poorly understood variability in the binding affinity among natural waters.

One approach to elucidating the source of this variability is to examine relationships between chemical and structural properties of DOM and its capacity to bind organic contaminants. For example, Gauthier et al. have reported for pyrene that the binding coefficients in natural waters correlated with the aromaticity of DOM in the water samples.[13]

It is possible to chemically fractionate DOM and determine if there are underlying similarities in binding affinities of functionally similar subcomponents of the total DOM. Nonionic macroporous sorbents, such as the Amberlite XAD resins, have been used to fractionate DOM into subcomponents based on the hydrophobicity and charge of the molecules.[14]

The objective of this chapter is to integrate our latest studies showing that fractions of DOM isolated using XAD-8 resin differ in their affinity for binding contaminants and to extend these observations to concern the bioavailability of selected model compounds in natural surface waters having a large variation in DOM concentrations.

MATERIALS AND METHODS

The natural water for the XAD-8 fractionation experiment was collected from a stream draining a peat deposit located in Hyde County, North Carolina,[15] natural waters for the benzo(a)pyrene association experiment were collected in North Carolina and Tennessee,[16] and natural waters used in the lake series experiment to study bioavailability of model compounds in different waters were collected in eastern Finland.[17]

Fractionation of DOM

The XAD-8 fractionation of DOM was modified from Leenheer and Huffman.[14] Water samples were filtered through precombusted glass-fiber filters (Whatman GF/C). Samples (150 mL) were acidified ($pH \leq 2$, concentrated H_2SO_4) and applied to the column of purified XAD-8 resin. The hydrophilic fraction (Hl) of the DOC is defined as that organic matter in the acidified water sample that was not retained by the column. The hydrophobic acid fraction (HbA) is defined as that organic matter eluted when the column was rinsed with 0.1 N NaOH. The hydrophobic neutral fraction (HbN) is defined as that organic matter retained by the XAD-8 and not eluted with base. The HbN fraction was extracted from the resin with methanol. Purifying procedures for different fractions to use them in further experiments are described by Kukkonen et al.[15]

Determination of Partition Coefficients

Equilibrium dialysis[3,5] was used to determine the K_p between model compounds and the fractions of DOM or DOM in the water samples. A filtered (Nuclepore, 0.22 μm) water sample (5 mL) was put into a dialysis bag (Spectra/Por 6, molecular weight cutoff of 1000 daltons) and placed in a glass jar containing an aqueous solution of a radiolabeled compound. Sodium azide (0.002%) was added to inhibit microbial activity. The jar was sealed with a Teflon-lined cap and shaken in the dark at 20°C for 4 days. At least three replicate determinations were made. Solutions inside and outside the dialysis bag were analyzed for ^{14}C activity using scintillation cocktail and a liquid scintillation counter. The outside concentration (C_o) is the freely dissolved organic pollutant, while the difference between the inside and outside concentration (C_p) is the pollutant bound to organic matter in the bag. K_p was calculated as

$$K_p = C_p/(C_o \times DOC) \qquad (7.1)$$

where DOC is the concentration of dissolved organic carbon (kg carbon/L).

Accumulation Experiments

Water samples were filtered (Nuclepore, 0.22 μm) and pH adjusted to 6.5 with 0.1 N NaOH and HCl. Aqueous concentrations of ^{14}C-labeled benzo(a)pyrene (BAP), 2,2′,5,5′-tetrachlorobiphenyl (2,2′,5,5′-TCB), 3,3′,4,4′-tetrachlorobiphenyl (3,3′,4,4′-TCB), naphthalene (NAPH), and ^{3}H-labeled dehydroabietic acid (DHAA) were 1, 2, 2, 5, and 70 μg/L, respectively—all below the published water solubility limits for each compound.

D. magna were obtained from a culture maintained at the Oak Ridge National Laboratory (Oak Ridge, TN) and were fed a mixture of trout chow, yeast, and *Cerophil* (for the study with the DOM fractions) or at University of Joensuu (Finland) and were fed a culture of *Monoraphidium contortum* (for the study with natural waters). Animals used in these experiments were 6–8 days old and did not have eggs in the brood chamber. Before exposures, daphnids were held for 1 hr in the clean control water to clear their gut contents. Groups of five *D. magna* were transferred to 100-mL glass beakers containing 50 mL of water sample containing one of the radiolabeled contaminants. Four replicate determinations were made for each sample. Beakers were kept in the dark at 20°C. After 24 hr, animals were removed from the water with a widemouthed pipet, collected on filter paper, briefly rinsed in 50 mL of distilled water, and blotted dry; all animals from each beaker were weighed together on a microbalance. The five animals were added to 10 mL of scintillation cocktail and analyzed for radioactivity. The radioactivity remaining in the exposure water was determined. Each experiment included a parallel control experiment using organic-free control water. The results are reported as a 24-hr bioconcentration factor (BCF) calculated as the ratio of the concentration of the pollutant in the animals (nanograms per gram wet weight) and in the water after the experiment (nanograms per milliliter), calculated from the specific activities of the compounds.

The fitting of regression lines shown in the figures as well as all statistical analyses (Student's t-tests, variance analyses, Pearson's correlations) were performed with SAS (Statistical Analysis System).[18]

Table 7.1. Characterization and Comparison of DOM in the Water Samples

Sample	DOC mg C/L	%HI	%HbA	%HbN	%TotHb	ABS_{270}	E_2/E_3	E_4/E_6	Reference
Hyde County sample	50	22	66	12	78	42.0	4.10	5.50	15
Brook Välioja	38.3	23	75	2	77	37.9	4.47	9.55	17
Brook Liuhapuro	32.2	20	77	3	80	43.4	4.49	12.4	17
Lake Ahvenlampi	20.4	30	63	7	70	29.5	4.71	3.64	17
Lake Louhilampi	18.1	18	61	20	81	32.7	4.78	8.20	17
Lake Makrijärvi	15.1	27	63	10	73	35.9	4.49	2.65	17
Lake Iso-Sormunen	13.5	22	51	27	78	30.6	4.88	3.33	17
Lake Melalampi	9.5	28	59	13	72	35.3	4.90	2.78	17
Lake Piimäjärvi	9.6	25	55	20	75	31.7	5.17	2.56	17
Lake Koitere	7.5	34	51	15	66	38.7	5.62	3.67	17
Lake Höytiäinen	7.5	29	40	31	71	36.9	5.63	4.00	17
Lake Riihilampi	6.8	32	44	24	68	35.1	4.63	2.50	17
Lake Viinijärvi (point 5)	8.4	32	39	28	67	26.4	5.88	5.00	17
Lake Iso-Hietajärvi	6.0	39	41	20	61	18.3	6.36	1.00	17
Lake Tammalammit	5.0	36	46	17	63	22.1	4.34	1.86	17
Lake Valkialampi	4.9	35	45	20	65	18.5	4.46	1.71	17
Lake Viinijärvi (point 1)	4.2	59	28	13	41	12.6	5.46	2.00	17
Lake Likolampi	4.9	48	22	30	52	10.8	4.25	1.50	17
Lake Miilunlampi	2.8	49	37	14	51	17.9	3.76	4.00	17
Lake Kakkisenlampi	2.0	49	38	13	51	22.4	3.29	1.43	17
Lake Kuorinka	3.0	42	19	39	58	13.4	3.79	1.33	
WB	—	51	27	22	49	15.5	na	2.78	16
P1	—	40	28	12	60	27.2	na	1.66	16
P2	—	59	30	11	41	12.1	na	1.53	16
P3	—	56	24	20	44	7.5	na	1.34	16
CH	—	40	40	20	60	27.5	na	2.76	16
H-0	—	40	48	16	60	17.8	na	3.22	16
H-15	—	35	51	14	65	20.0	na	11.1	16
H-30	—	32	26	42	68	2.9	na	1.40	16
H-100	—	31	24	42	69	3.1	na	1.13	16
B-0	—	22	60	17	78	38.0	na	4.32	16
B-50	—	34	36	31	66	9.2	na	1.28	16
ALD (Aldrich HA)	—	30	57	13	70	63.0	na	5.58	16

Notes: %HI = percentage of DOC determined to be hydrophilic.
%HbA = percentage of DOC determined to be hydrophobic acids.
%HbN = percentage of DOC determined to be hydrophobic neutrals (HbN = DOC – HI – HbA).
%TotHb = percentage of DOC determined to be hydrophobic (TotHb = DOC – HI).
ABS_{270} = absorptivity at 270 nm (units of L/mg C × cm × 10^3).
E_2/E_3 = ratio of absorbances at 250 to 365 nm.
E_4/E_6 = ratio of absorbances at 465 to 665 nm.
na = not analyzed.

CHARACTERIZATION OF DOM IN NATURAL WATERS

Percentages of hydrophilic (Hl), hydrophobic acid (HbA), and hydrophobic neutral (HbN) fractions in experimental waters and some UV-VIS spectroscopic characterizations like ABS_{270} (1000 x absorbance at 270 nm/DOC), E_2/E_3 (absorbance ratio A250/A365) and E_4/E_6 (absorbance ratio A465/A665) are reported in Table 7.1. The spectroscopic characterization of isolated DOM fractions is shown in Table 7.2. According to Chen et al., the high E_4/E_6 ratio

Table 7.2. Spectroscopic Characteristics of Different DOM Fractions in Hyde County Water Sample

Fraction	ABS_{270}	E_2/E_3	E_4/E_6
Hydrophobic acids	59	4.1	7.5
Hydrophobic neutrals	4	4.7	3.4
Hydrophilic compounds	13	5.6	3.1

Source: Kukkonen et al.[15].
Note: Spectra were run at pH 6.0 using samples having DOC concentrations of 10 mg C/L.

can be related to high molecular weight.[19] Schnitzer reported that the E_4/E_6 value for fulvic acids and humic acids extracted from soils are in the range of 7.6 to 11.5 and 3.8 to 5.8, respectively.[20] According to De Haan, fulvic acids from strongly humified and oligotrophic waters are characterized by a relatively low E_2/E_3 ratio (about 4).[21]

The spectroscopic characteristics of the isolated fractions (Table 7.2) suggest that they differ substantially from each other in chemical composition. Differences in absorptivity at 270 nm (ABS_{270}) reflect absorbance of pi-pi* transitions in substituted benzenes and most polyenes and have been related to the aromatic content of isolated soil humic acids.[13] The observed spectral differences suggest that the HbA fraction is enriched in aromatic content, relative to the HbN fraction. The Hl fraction is intermediate between the two other fractions.

Table 7.1 shows the variation in natural waters—from dark-colored brook waters having an extremely large HbA portion to clear water lakes having low DOC concentrations and a small HbA portion. The correlations in the Finnish water series between percentage of HbA and DOC concentration ($r = 0.84$, $p = 0.001$), ABS_{270} ($r = 0.82$, $p < 0.001$) and hydrogen:carbon atomic ratio ($r = -0.81$, $p < 0.001$) and in the U.S. water series between percentage of HbA and ABS_{270} ($r = 0.75$, $p < 0.05$) indicate the dominant role of the HbA fraction in the natural waters. These data suggest that the HbA fraction is enriched in aromatic content, as can be expected knowing that the HbA fraction is mainly humic compounds (i.e., fulvic and humic acids). On the other hand, the HbN fraction showed a very low absorptivity at 270 nm (Table 7.2), and the same can be seen in the natural water series. The percentage of HbN fraction and ABS_{270} correlated negatively in the whole data set ($r = -0.52$, $p < 0.05$).

BINDING OF CONTAMINANTS BY DOM

Binding of Contaminants to DOM Fractions

The hydrophobic fractions of the Hyde County water sample have the greatest affinity for binding BAP (Figure 7.1).[15] The HbA fraction has a significantly higher K_p ($p < 0.05$) than the HbN fraction. The Hl fraction has much lower affinity for binding BAP. Amy and Liu have shown similar results for

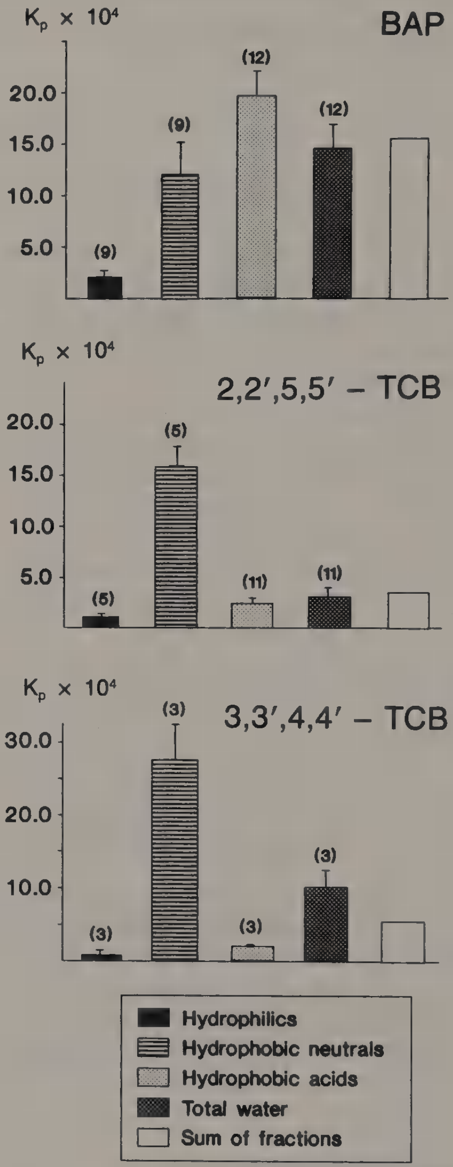

Figure 7.1. Partition coefficients (K_p) of total (unfractionated) Hyde County water sample and different DOM fractions for BAP, 2,2′,5,5′-TCB, and 3,3′,4,4′-TCB. Bars indicate the mean values (± SD), and the number in parentheses is the number of replicates. Data from Kukkonen et al.[15].

phenanthrene in the XAD-8 fractions of DOM.[22] The K_p of the total water appears to reflect the sum of the binding affinities of the individual fractions, with little indication of interactive effects among the fractions. The measured K_p for the total water agrees very well with a cumulative K_p calculated from the sum of the K_p for the individual fractions ($K_{p(i)}$) and the relative contribution (f_i) of the i^{th} XAD fraction to the total DOC:[15]

$$\text{Sum } K_p = (K_{p(HL)}\, f_{HL}) + (K_{p(HBA)}\, f_{HBA}) + (K_{p(HBN)}\, f_{HBN}) \qquad (7.2)$$

The relative binding affinities of the DOC fractions for 2,2′,5,5′-TCB and 3,3′,4,4′-TCB (Figure 7.1) exhibited a different pattern from that observed for BAP.[15] The HbN fraction had the highest affinity for binding TCB. The sum of K_p's of different fractions with TCB also agreed with the measured K_p value in the total water. This different affinity for binding to isolated XAD-8 fractions of DOM was attributed to differences in the electron densities of DOM fractions and model compounds. BAP is an electron-rich compound and will have a tendency to donate its electrons via charge transfer mechanism to electron-deficient compounds like HbA. Conversely, PCBs are electrophiles and may be attracted to the richer electron densities of HbN material. The K_p values for NAPH were much lower (in total water: $K_p = 1150 \pm 476$; in HbA: $K_p = 785 \pm 225$) than for BAP, 2,2′,5,5′-TCB, and 3,3′,4,4′-TCB.

Binding of Contaminants to DOM in Lake Waters

The partition coefficients (K_p) for BAP and 3,3′,4,4′-TCB in different natural waters are shown in Figure 7.2.[17] The K_p values for BAP were similar to those reported for natural DOM samples.[9,23] Also, the K_p values for 3,3′,4,4′-TCB agree well with the published partition coefficients for the same or similar compounds.[4,23] For BAP it was possible to measure the K_p value for every water sample, but for the other model compounds, especially NAPH and DHAA, which have much lower K_p values (~300–6000)—that is, not much interaction with DOM—the dialysis method is not sensitive enough to obtain accurate measurements in the water samples with low DOC concentration.[17]

There were strong direct relationships between the K_p values of BAP and hydrophobic acid content of the natural waters ($r = 0.77$, $p < 0.001$, for Finnish waters;[17] $r = 0.74$, $p < 0.01$, for McCarthy et al. data[16]) and a good negative correlation between the K_p values of BAP and the hydrogen:carbon ratio of DOM ($r = 0.76$, $p < 0.001$).[17] The absorptivity at 270 nm (ABS_{270}) gave the best correlation with the K_p values for BAP ($r = 0.87$, $p < 0.001$, from Kukkonen and Oikari;[17] $r = 0.94$, $p < 0.001$, from McCarthy et al.[16]). In Figure 7.3, these data are plotted together. The relationship between ABS_{270} and log K_p values of BAP is similar in these two sets of natural waters. The results of BAP in these two studies also agree well with the results reported by Gauthier et al.,[13] who also showed an excellent correlation between both aromatic content and absorptivity at 270 nm of humic materials extracted from

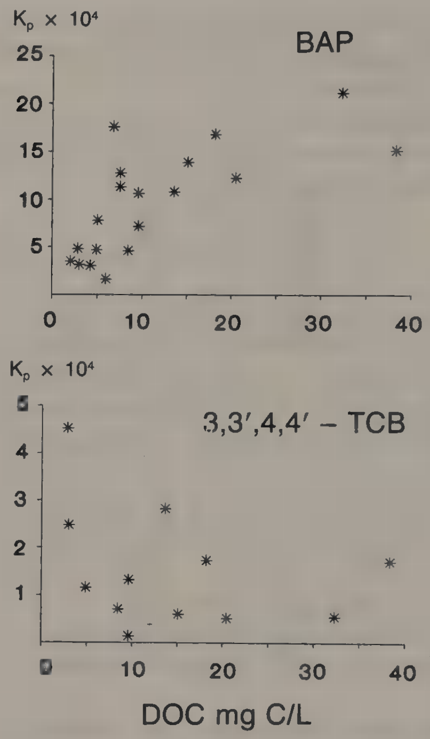

Figure 7.2. Partition coefficients (K_p) of water samples in lake series from Finland for BAP and 3,3′,4,4′-TCB. Each point indicates the mean value of four replicates. Data from Kukkonen and Oikari.[17]

soils and sediments and the partition coefficient for binding of pyrene to these samples. On the other hand, the K_p values for BAP and the percentage of HbN fraction had slight negative correlation ($r = -0.53$, $p < 0.1$, from McCarthy et al.;[16] $r = -0.40$, $p < 0.1$, from Kukkonen and Oikari[17]), which is in accordance with the results obtained in the study with different fractions.[15] Taken together, these studies suggest that observed differences in affinities of BAP and also some other organic pollutants between different water sources are, at least partly, explained by different percentages of hydrophobic acids and the aromaticity of DOM.

EFFECTS OF DOM ON BIOAVAILABILITY OF ORGANIC CONTAMINANTS

Bioavailability of Contaminants to *D. magna* in DOM Fractions

Accumulation of BAP by *D. magna* in DOM fractions was reduced by increasing the concentration of DOC (Table 7.3). The BCF value for BAP in the organic-free control water was significantly higher than BCFs for all con-

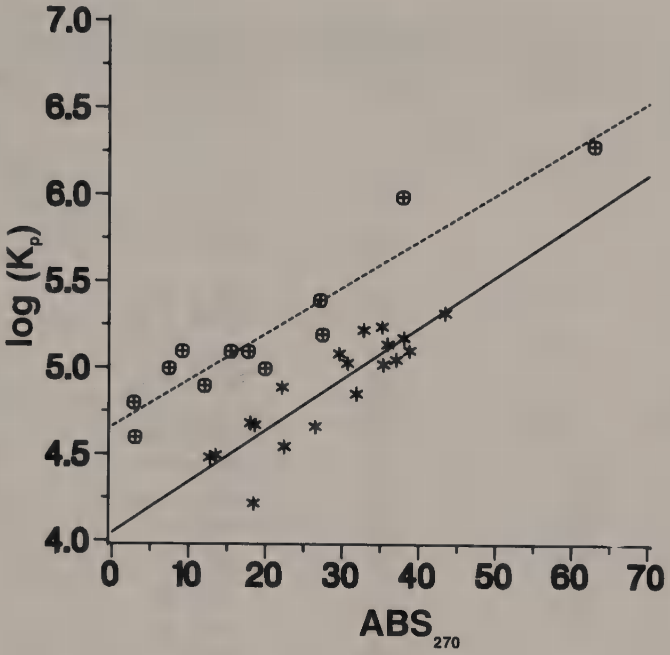

Figure 7.3. The log K_p for binding BAP to different sources of DOM is directly related to the ABS_{270} of the water. *Stars* and the *solid line* are for surface water samples from eastern Finland,[17] and *circles* and the *dashed line* are for surface and groundwater samples from the United States.[16]

centrations of DOM fractions ($p < 0.05$). The HbA fraction had the greatest effect on the bioavailability of BAP, and the HbN and Hl fractions also reduced the bioaccumulation of BAP compared to the control (Table 7.3), but the effect is not as great as for the HbA fraction. Approximately five times more organic carbon from the Hl fraction was required to reduce the BCF to the extent observed for the HbA fraction or the total water. The potency of the HbN fraction is intermediate to the two other fractions.[15]

The HbN fraction was more effective in reducing the accumulation of 2,2′,5,5′-TCB than the other carbon sources, although the effect is not significantly different from that for the HbA fraction at the same DOC concentration. The decrease in the BCF due to this fraction was much less than was anticipated on the basis of its affinity for binding 2,2′,5,5′-TCB (Figure 7.2). A "biological K_p" for 2,2′,5,5′-TCB was back-calculated based on the reduction in BCF with the HbN fraction; this value (approximately 6.4×10^4) was lower than the K_p measured using equilibrium dialysis, but it is still higher than those determined for the other fractions or for the total water.[15]

The bioaccumulation of NAPH in *D. magna* was much less than for the other compounds, and the accumulation data agree with the K_p measurements

Table 7.3. The Bioconcentration Factors for Benzo(a)pyrene, 2,2′,5,5′-Tetrachlorobiphenyl, and Naphthalene in the Different DOC Fractions

Chemical	Fraction	[DOC]	Observed BCF	Predicted BCF
BAP control		0	5990 ± 679	—
	HbA	1	4792 ± 448	5004
		2	3765 ± 721	4296
		5	3116 ± 334	3017
		15	2151 ± 180	1514
		25	1744 ± 214	1011
	HI	5	5103 ± 535	5408
		10	3565 ± 376	4930
	HbN	5	4430 ± 703	3767
		15	2608 ± 214	2162
22′55′-TCB	control	0	6543 ± 844	—
	HbA	5	6090 ± 713	5834
		10	4669 ± 963	5263
	HI	10	6028 ± 1109	5910
	HbN	10	4164 ± 431	2526
NAPH	control	0	37 ± 4	—
	HbA	5	47 ± 4	37
		10	44 ± 5	36
		25	36 ± 5	35
		50	33 ± 4	34
	HI	10	37 ± 4	36
	HbN		not measured	

Source: Kukkonen et al.[15]

Notes: The numbers are means (± standard deviation) of four replicate accumulation measurements. DOC concentrations are mg C/L. The predicted value of the BCF (Equations 7.3 and 7.4), based on the fraction of the pollutant calculated to be freely dissolved from equilibrium dialysis experiments, is also indicated.

in that NAPH does not appear to have much interaction with dissolved organic material compared to BAP and 2,2′,5,5′-TCB.[15]

Bioavailability of Contaminants to *D. magna* in Natural Waters

Accumulation of model compounds by *D. magna* was reduced by increasing the concentration of DOC also in the natural water series or in the diluted Hyde County water series (Figure 7.4). The relationship between DOC concentration and BCFs is similar in the series of different natural waters from Finland,[17] in diluted concentrations of Hyde County water,[15] and in some water samples or natural humus preparations that are diluted down to different DOC concentrations with control water.[9]

The BCF values for model compounds in the organic-free control water were the same or significantly ($p < 0.05$) higher than BCFs for water samples from Finland,[17] except in the case of NAPH, where three natural waters having low DOM concentration (2–4 mg C/L) revealed significantly ($p < 0.05$) higher BCF values than the control water. The increase of bioaccumulation of NAPH

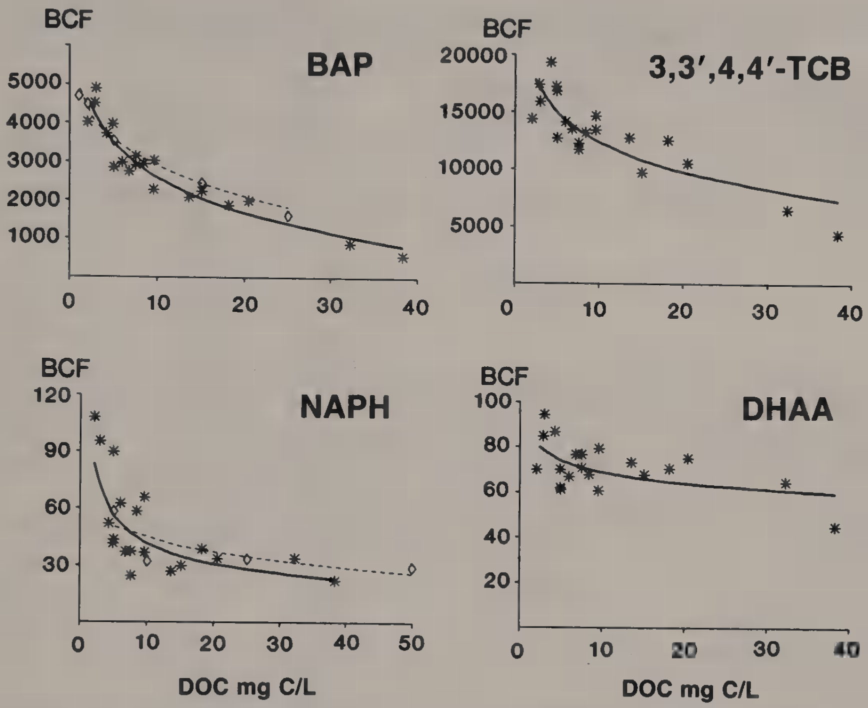

Figure 7.4. The bioconcentration factors (BCFs) of model compounds in natural surface waters from eastern Finland having different DOC concentrations *(stars and the solid line)*[17] and in diluted Hyde County water sample *(diamond and the dashed line)*.[15] Each point indicates the mean value of four replicates, and the lines are regression lines fitted to data points. Note that the control value (no DOM in water) for NAPH is 37.

in the waters having low natural DOC concentration compared to organic-free control water (DOC ≤ 0.3 mg C/L) is an interesting phenomenon, and similar data have been reported earlier; for example, the bioavailability of methylcholanthrene to *D. magna* was increased by Aldrich humic acid (concentration, 2 mg DOC/L) in the study by Leversee et al.,[24] but McCarthy et al. showed opposite results in their study with *D. magna*.[10]

The bioavailability of DHAA was reduced by the DOM, even in the less DOM-enriched waters, but the effect of increasing DOM concentration is not as clear as it is for other model compounds used (Figure 7.4).[17] DHAA has a low K_p value,[17] and it is a weak organic acid (pK_a 5.7),[25] which is more than 50% ionized at the experimental pH of 6.5.

The measured BCF values correlated with the percentage of hydrophobic acids, absorptivity at 270 nm, and hydrogen:carbon ratio even in the case of NAPH and DHAA (Table 7.4). This result by Kukkonen and Oikari[17] indicates that in addition to the total DOM concentration, the quality of DOM also affects very clearly the bioavailability of organic xenobiotics. It is impor-

Table 7.4. Statistical Relationships between BCF Values of Model Compounds and Some Chemical Parameters of DOM, Based on Simple Linear Regression Analysis on Pairs of Variables

Dependent Variable	Independent Variable	Slope ($\pm$SE)	Intercept ($\pm$SE)	r^2
Log_{10}(BCF of BAP)	DOC	–0.022 (0.001)	3.66 (0.02)	0.94
BCF of BAP	%HbA	–67.4 (7.9)	6139 (407)	0.81
	H/C atomic ratio	2757 (408)	–546 (517)	0.73
	ABS_{270}	–93.6 (18.4)	5481 (549)	0.60
Log_{10}(BCF of 3,3′,4,4′-TCB)	DOC	–0.014 (0.001)	4.25 (0.02)	0.86
BCF of 3,3′,4,4′-TCB	%HbA	–190 (29.4)	22214 (1475)	0.70
	ABS_{270}	–289 (52.0)	21094 (1516)	0.63
	H/C atomic ratio	7452 (1740)	3954 (2225)	0.50
BCF of Naphthalene	H/C atomic ratio	75.1 (11.7)	–39.4 (14.4)	0.71
	DOC	–54.4 (11.7)	100 (11.5)	0.56
	ABS_{270}	–1.69 (0.47)	97.6 (14.0)	0.43
	%HbA	–1.08 (0.31)	103 (15.9)	0.42
Log_{10}(BCF of DHAA)	DOC	–0.004 (0.001)	1.89 (0.02)	0.35
BCF of DHAA	H/C atomic ratio	20.8 (5.56)	45.5 (7.11)	0.44
	%HbA	–0.38 (0.13)	89.4 (6.66)	0.31
	$r^2 < 0.2$ for ABS_{270}			

Source: Kukkonen and Oikari.[17]

Notes: The units for the variables are as described in Table 7.1. The regressions between BCFs and DOC are plotted in Figure 7.4.

tant to note that the bioavailability of NAPH and DHAA correlate similarly with the chemical parameters of DOM, as is the case with more lipophilic compounds.

The observed data for bioaccumulation of BAP in different DOM fractions and natural waters were compared to those which would be predicted based on the assumption that BAP bound to the DOM is unavailable for uptake by the organism; that is, bioaccumulation in water containing DOM will be proportional to the fraction of the contaminant that is freely dissolved (f_{free}):

$$\text{predicted BCF in presence of DOM} = \text{control BCF} \times f_{free} \tag{7.3}$$

where f_{free} is calculated from the measured K_p and DOC concentration of each sample:

$$f_{free} = 1/(1 + K_p \times \text{DOC}) \tag{7.4}$$

where DOC concentration is expressed as kg carbon/L. The measured BCF values of BAP agreed well with the predicted BCF values for BAP from Equation 7.3 in both experiments with DOM fractions and natural waters (Figure 7.5). The 95% confidence limits for this regression overlaps the 1:1 line, which is predicted by the hypothesis expressed in Equation 7.3 and is consistent with similar results for BAP in *D. magna,*[10] *Pontoporeia hoyi,*[26] and rainbow trout.[11] The study by Kukkonen and Oikari extends these observa-

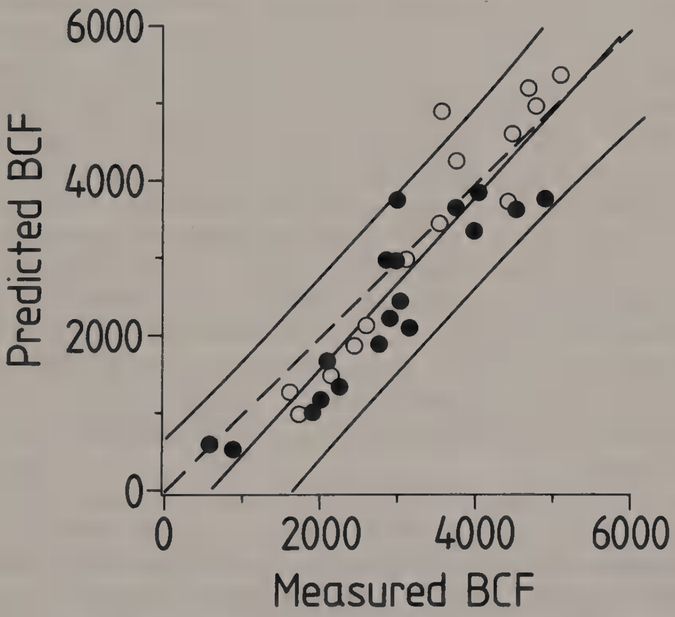

Figure 7.5. The predicted BCF of BAP (from Equation 7.3) plotted against the measured BCF in the water sample. *Filled-in circles* represent the natural water series from Finland;[17] *open circles*, the experiment with different DOM fractions.[15] The 95% confidence interval *(solid curves)* of the regression line *(solid line)* overlaps the 1:1 line *(dashed line)* predicted by the hypothesis that only the freely dissolved BAP is available for *D. magna*.

tions to a series of natural waters having both quantitatively and qualitatively different DOM content and provides additional confirmation that the effects of natural DOM on bioaccumulation of BAP can be predicted from physicochemical measurements of K_p's.[17] But we have to keep in mind that this conclusion works well with compounds like BAP, which is highly lipophilic and therefore has a high affinity to the DOM; it appears that a high enough or strong enough interaction between the xenobiotics and DOM is needed before this kind of prediction can be made. Also the best method to measure K_p values may vary from one compound to another. However, Black and McCarthy got similar results for BAP and 2,2′,5,5′-tetrachlorobiphenyl by measuring the K_p value of 2,2′,5,5′-tetrachlorobiphenyl by a reverse-phase separation method and using Aldrich humic acid,[11] which has a higher affinity to bind xenobiotics than natural DOM.[16]

ENVIRONMENTAL IMPLICATIONS

These reviewed papers have extended previous observations on the role of natural DOM in binding hydrophobic organic compounds and, more importantly, in altering their availability for uptake into biota in different types of natural waters. There is a large degree of variability in the affinity of DOM from different sources of water to bind hydrophobic organic compounds. The total concentration of DOM in a water is not always a good predictor of the capacity of that water for binding organic xenobiotics. It is shown in these papers that the qualitative differences in the nature of organic material from different sources have a large effect on its affinity for binding lipophilic organic xenobiotics. This kind of chemical variability can also explain the results where the K_p values for binding xenobiotics differed by orders of magnitude for DOM from natural waters taken from different locations.[3,8,23,27] These findings may also give us a tool to predict the possible interactions between pollutants and DOM in water, and perhaps even the effects of natural DOM on the transport and bioavailability of contaminants. In the case of BAP, this seems to be quite possible, but for other compounds there is a need for further research.

The total concentration of DOM in the natural waters is one of the main factors controlling the bioavailability of highly lipophilic xenobiotics ($K_{o/w} > 10^4$), but in addition to the quantity, the quality of DOM (i.e., aromaticity and portion of hydrophobic acids) can also play an important role.

An ecotoxicologically interesting result was the increased bioavailability of NAPH in waters having low DOM concentrations compared to the organic-free control water. It is important to confirm this result with further experiments, and then perhaps also to find some explanations for the results concerning increased toxicity of some organic xenobiotics in natural waters.[12,28]

ACKNOWLEDGMENTS

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CHAPTER 8

Solvent-Filled Dialysis Membranes Mimic Bioaccumulation of Pollutants in Aquatic Environments

Anders Södergren

INTRODUCTION

It is generally accepted that the concentration of persistent pollutants in gill-breathing aquatic animals is mainly controlled by equilibrium partitioning; that is, the uptake and depuration of the pollutants is a function of their exchange through the gills and across the body surface before reaching equilibrium levels between the ambient water and the body lipids.[1-3] The levels of the pollutants in organisms belonging to low trophic levels (e.g., plankton, crustacea, shellfish, fish) thus depend primarily on the levels of the pollutant in the water. The uptake of additional quantities through the ingestion of contaminated food does not greatly influence the total concentration attained in the organism. At high trophic levels, however, an age-dependent accumulation is often found.[4,5]

It has previously been shown that passive samplers consisting of solvent-filled membranes, which imitate body lipids, will accumulate organochlorine pollutants in a similar manner to gill-breathing organisms.[6,7] Monitoring of lipophilic, persistent pollutants in aquatic environments with such samplers is therefore an alternative to the determination of the residues accumulated by organisms.[8] To compare uptake patterns of chlorinated and nonchlorinated pollutants, solvent-filled membranes were exposed to polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAH) in the laboratory and in the field, and their properties during long-term exposure were followed. In addition, the rate of depuration and effects of lipids on the capacity of the membrane to accumulate the pollutants were studied.

MATERIAL AND METHODS

Dialysis membranes (Spectra/Por 3 and 6, cutoff 1000 Da) were filled with about 4 mL of n-hexane and exposed to water in the laboratory or buried in the sediment in the field. The use of membranes with a cutoff of 1000 prevents substances of higher molecular weight from diffusing through their walls, thereby simplifying the cleanup procedure.

The effect of different volumes of solvent in the membranes was followed by varying the amount of hexane from 0.6 to 4.8 mL while keeping the membrane area constant. To study the influence of lipids on the rate of uptake, mixed triglycerides (glyceryl-1,2-myristate-3-palmitate) were added to the solvent of the membranes in proportions of 0.1, 1.0, 5.0, and 10.0%.

The membranes were exposed to a constant level of the substances, which were added to the water in a continuous-flow system. The substances added were creosote, which is used as a wood preservative agent, and Clophen A 50, a mixture of polychlorinated biphenyls (PCBs) with an average chlorination of 50%. The sediment in the field was "naturally" contaminated via effluents from a creosote impregnating site. Among the PAH in the creosote mixture, only fluorene, phenanthrene, and fluoranthene will be discussed here.

Since a relationship is likely to exist between the surface area and the rate of uptake of lipophilic residues, the area of the membranes was held constant at about 1200 mm^2. The membranes were either washed and sonicated in hexane or washed with distilled water before filling to remove the sulfur left from the manufacturing process and the preservatives (usually mixtures of glycerols) that protect the membranes during storage. After being filled with the solvent, the membranes were stored until use in polypropylene bottles filled with distilled water. After exposure the bottles with the membranes were filled with water or sediment from the exposure site before being returned to the laboratory.

Spectra/Por polypropylene closures were used to tie the membrane tubings, and they were protected from being damaged during their exposure in the field by metal holders.[8] The holders were suspended in the water or buried in the sediment.

Before use, a PCB congener (2,2′,5,6′-tetrachlorobiphenyl, IUPAC #53) was added to the solvent in the membranes for use as an internal standard. The recovery of the congener served as a check of the functioning of the membrane.[8]

The resistance to microbial degradation was studied by storing two solvent-filled membranes for about 1 year in a suspension of sewage sludge at room temperature. The effect of the solvent on the properties of the membrane was evaluated by comparison with membranes filled with distilled water.

Processing of the samples was simplified since no cleanup of the solvent in the membranes was normally necessary. Depending on the exposure situation, the solvent was evaporated to 1 or 0.25 mL before separation and quantification by gas chromatography. The analytical procedure has previously been described.[6]

In accordance with the definition of the bioconcentration factor (BCF), the capacity of the solvent-filled membranes to accumulate substances was expressed as a membrane concentration factor (MCF = concentration in the membrane/concentration in the water or in the sediment).

RESULTS AND DISCUSSION

The two membranes filled with solvents were resistant to degradation since no solvent was lost after long-term exposure in a sewage sludge mixture (Figure 8.1). The increase in weight of the membranes was mainly a result of adsorption of humic material onto the membrane wall. Damage in the form of shallow cavities was noted, but in no case had holes developed. The results indicate that the solvent partially impregnates the membrane wall and inhibits

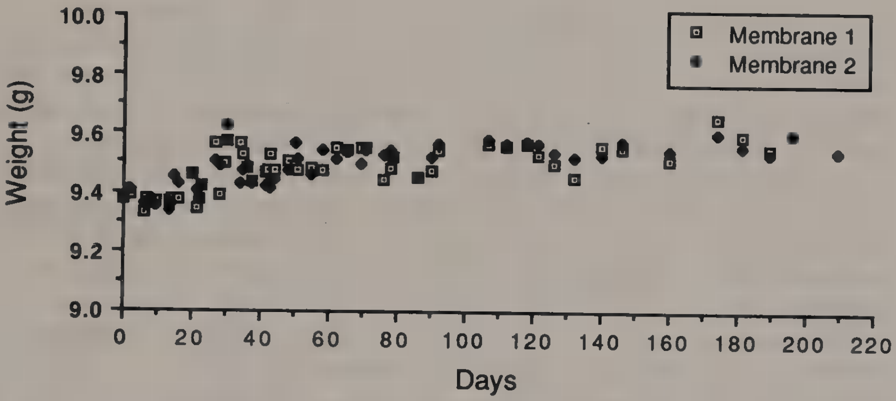

Figure 8.1. Changes of the content of solvent (hexane) in two dialysis membranes stored in water at about 20°C in the laboratory.

Table 8.1. Uptake of PCBs by Dialysis Membranes (Area 1200 mm^2) Filled with Different Volumes of n-Hexane

Volume (mL)	PCBs (ng/ml)	
	120 hr	12 days
0.6	1.04	284
0.6	1.21	277
0.6	0.88	260
1.2	0.92	272
1.2	0.88	264
1.2	1.32	255
4.8	1.12	264
4.8	1.03	238
4.8	1.10	230

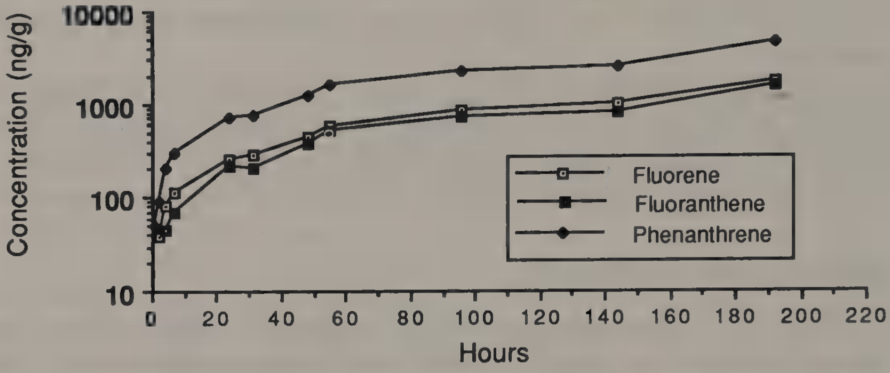

Figure 8.2. Uptake of PAH by solvent-filled dialysis membranes exposed in continuous-flow systems (mean of 4 replicates). The mean concentration of fluorene in the water was 0.13 μg/L (SD 0.06), phenanthrene 0.36 μg/L (SD 0.19), and fluoranthene 0.16 μg/L (0.09) (n = 6).

microorganism growth, presumably by its toxic effects. Membranes thoroughly washed in distilled water and hexane before adding the solvent have been shown to be less affected than membranes washed only in hexane.[8] The solvent-filled membranes may thus be placed for several months in receiving waters, imitating a natural exposure situation of an aquatic organism.

Decreasing the volume of hexane in the membranes by eight times while keeping their area constant resulted in limited changes in their rate of uptake of PCBs (Table 8.1). The amount of solvent in the membrane therefore does not seem to be a crucial factor in determining the functioning of the membrane.

PAH compounds were taken up by membranes buried in sediment or exposed to contaminated water in the laboratory. After 200 hr of exposure in

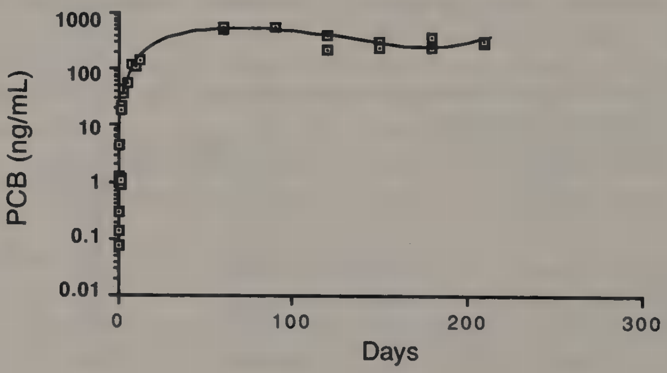

Figure 8.3. Uptake and depuration of PCBs from solvent-filled dialysis membranes. The exposure (7.7 μg/L) was stopped after 12 days and the membranes placed into clean running water. One dot represents one membrane.

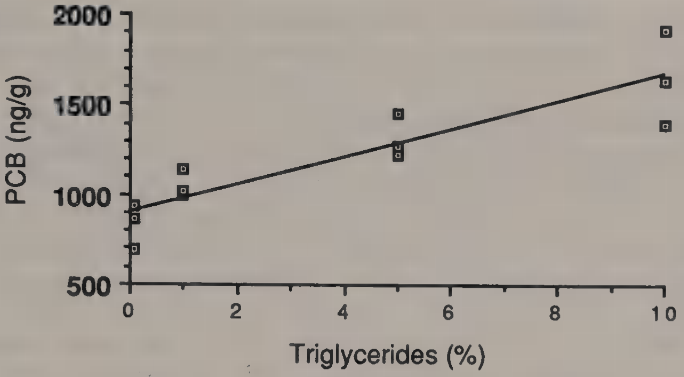

Figure 8.4. Effect of triglycerides added to the solvent-filled dialysis membranes on the uptake of PCBs from water. The membranes were exposed for 12 days. One dot represents one membrane.

the water, the levels in the membranes were still increasing and no steady state was approached, since there existed a significant difference in the uptake of all the compounds at 144 and 192 hr (Student's t-test, $P < 0.005$, Figure 8.2). This is in contrast to the uptake pattern of more lipophilic residues such as PCBs, where an apparent steady state was achieved after about 100–200 hr when exposed in the water[6,7] or in the sediment.[8] At the end of the experiment, fluorene was concentrated in the membranes 231 times, phenanthrene 174 times, and fluoroanthene 225 times relative to their levels in the water. In comparison to the PAH, the MCF of membranes exposed for PCBs in water is higher (about 275).[6] The more lipophilic character of the organochlorine residues compared to the PAH may explain this difference in rate of uptake and accumulation.

The levels of fluorene in membranes kept under identical conditions for 8 days in PAH-contaminated sediment show that it is possible to produce membranes with similar properties to absorb those substances from the interstitial water. The membranes accumulated fluorene (1702 ng/g, SD 156, $n = 6$) at levels approximately 150 times greater than those in the sediment pore water. The membranes thus seem to absorb the compounds regardless of whether they are exposed to water or buried in sediment. Since no particles can penetrate the membrane, the results show that the compounds taken up are present in a dissolved state in the water and in the sediment pore water.

Once taken up, the PCBs left the solvent-filled membranes slowly (Figure 8.3). After a rapid initial uptake the level stabilized at about 500 ng/mL and decreased only slightly after being placed in clean running water. This is a pattern shared with several aquatic organisms. For example, it has been shown in clearance experiments with various lipophilic, persistent compounds that the rate of uptake by organisms is often fast in comparison to the depuration.[9,10] However, the pattern of elimination seems to be determined by several factors since experimental conditions such as dose and the exposure time have been found to influence the rate of depuration from the organisms. Use of solvent-filled membranes may facilitate clarification of the mechanisms involved since no transformation or degradation of the substances will occur, thus enabling the study of the passive phases in the elimination processes. The solvent-filled membrane can be described kinetically as a homogeneous compartment, which means that uptake and elimination time constants are proportional to the volume of water passing the membrane. These time constants can be broken down into times attributable to water-phase and lipid-phase diffusion flow processes, which can be determined using the solvent-filled membranes.

Addition of triglycerides to the solvent of the membranes changed their capacity to accumulate pollutants (Figure 8.4). Increasing the proportion of triglycerides from 0.1 to 10% doubled the accumulation of the PCB compounds in the membranes but also increased the variability of the results. It is well established that lipids are important in the bioaccumulation process, governing the partitioning of the pollutants between the organism and the water.[9] These results show that triglycerides are of significance in accumulation of lipophilic residues. Since no mechanisms other than diffusion and partitioning are involved when using the membranes, these experiments suggest that the properties of the lipids involved, and hence the partitioning mechanism, are the principal factors determining the rate of uptake and accumulation. The specific details behind these processes may be examined by using the membranes with specific lipids, lipid mixtures, and finally whole body fluids. The individual effects of chosen parameters can be isolated since influences from body functions (degradation, depuration, inhomogeneous distribution between different body fluids, etc.) normally encountered in organisms will not complicate interpretation of the results.

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B. Bioaccumulation

CHAPTER 9

Bioaccumulation of Molecular Markers for Municipal Wastes by *Mytilus edulis*

Paul M. Sherblom and Robert P. Eganhouse

INTRODUCTION

Municipal wastewaters serve as a vehicle for the release of anthropogenic contaminants to the coastal environment. Estimating the wastewater contribution to an organism's tissue burden of specific contaminants (e.g., PAH, PCBs) is often complicated due to alternate potential sources (e.g., industrial waste, stormwater runoff, atmospheric deposition). One means of characterizing the role of municipal wastewaters as a pollutant source to sediment, suspended particles, and biological tissues is through the use of source-specific marker compounds. Two types of such markers that have been used to evaluate waste impacts on sediments and particles are (1) the fecal sterol, coprostanol, and (2) the long-chain linear alkylbenzenes (Figure 9.1).

Coprostanol is produced via microbial hydrogenation of the double bond between carbons 5 and 6 in cholesterol. Hydrogenation at this point can give rise to two possible structures: coprostanol (5β-cholestan-3β-ol) and cholestanol (5α-cholestan-3β-ol). While cholestanol has other sources in the environment and is the thermodynamically favored product, it appears that production by enteric bacteria in higher animals is the dominant source of coprostanol.[1] Coprostanol has been widely used as an indicator of sewage contamination.[1,2] Several authors have also investigated the relationship between organic contaminant and coprostanol concentrations in the marine environment relative to municipal waste inputs.[3–7]

The long-chain linear alkylbenzenes (LABs), a suite of 26 secondary phenylalkanes with alkyl side chains of 10 to 14 carbons, are produced industrially as precursors to the anionic surfactants, linear alkylbenzene sulfonates (LAS). These compounds have been identified in municipal wastes,[8–13] marine sediments,[14–17] and suspended and sinking marine particles.[8,14,18] The presence of LABs in wastewater effluents is thought to result from incomplete sulfonation and carryover in detergents, and/or from desulfonation of LAS.[14]

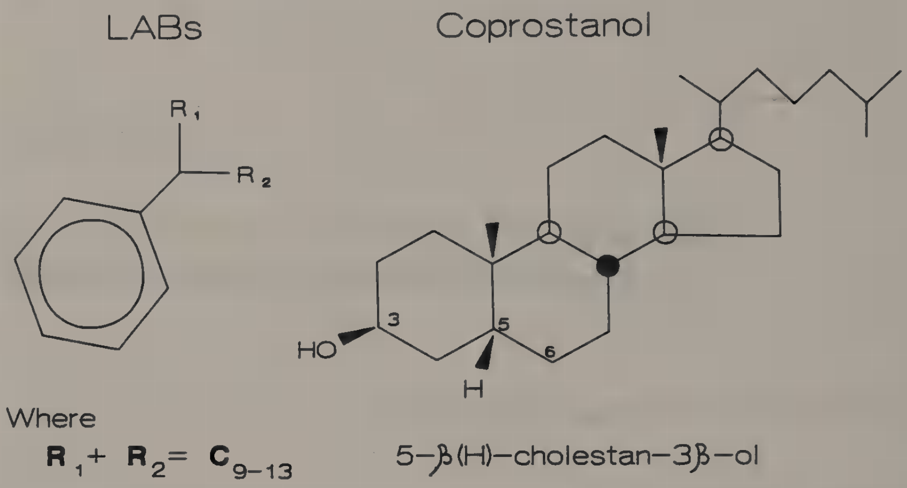

Figure 9.1. Structures of the municipal waste markers: the long-chain linear alkylbenzenes (LABs) and coprostanol.

The LABs exhibit a range of physicochemical properties spanning those of many hydrophobic organic contaminants (e.g., PAH, PCBs).[19] This enhances their potential utility for mimicking the transport and fate of other hydrophobic organics discharged in municipal wastes.

The marine bivalve, *Mytilus edulis*, has been widely used as a biomonitor of coastal pollution.[22,23] Mussels are well suited to this role because they are essentially stationary, filter large quantities of water, and have a limited ability to metabolize xenobiotics.[24] Bioaccumulation of hydrophobic organic substances is thought to be dominated by partitioning into tissue lipids.[25] Thus, changes in the storage of lipids may be reflected in the tissue burden of hydrophobic contaminants. Whether changes in tissue weight and lipids (related to the organism's reproductive cycle) have effects on the body burden of hydrophobic organics is a matter of discussion.[26,27] Other factors that may affect a comparison of samples across a time series include potential changes in animal size and/or tissue wet weight (nonlipid related).

Both of the molecular markers described above have been identified in organisms.[28–34] Zollo et al. reported the presence of coprostanol in tunicates collected in the Bay of Naples (Italy), but they did not speculate on whether it was biosynthesized or accumulated.[28] Matusik et al. determined the presence of both coprostanol and epicoprostanol (5β-cholestan-3α-ol) in samples of *Mercinaria mercinaria* collected in sewage-contaminated waters.[29] O'Rourke sampled lobsters, clams, and polychaetes from both sewage-impacted and nonimpacted sites.[30] She found coprostanol to be present only in intestinal tissues of organisms collected from contaminated sites. Since mussels are usually analyzed as whole organism composites, the presence of coprostanol might be due to sewage-derived particles lodged in the gut of the organism rather than bioaccumulation.

Werner and Kimerle explored the bioaccumulation and elimination of LABs by fish (*Lepomis macroshirus*) using laboratory exposures.[31] Their results indicated that tissue concentrations were modified by metabolism and/or elimination of the LABs by the fish. However, Albaigés et al. suggest that the presence of LABs in liver samples of fish (*Micromesistius poutassou*) collected off the coast of Spain is indicative of their environmental persistence.[32] Yasuhara and Morita, investigating "volatile" organic components in mussels, report the presence of several alkylbenzenes with alkyl chains of 10 to 13 carbons in length.[33] Murray et al. found the full suite of LABs in mussels collected in Port Phillip Bay (Australia).[34]

The tissue distributions and bioaccumulation rates of the marker compounds need to be evaluated before they can be used to estimate the tissue burdens of other hydrophobic pollutants that may be due to municipal waste discharges. Thus, we chose to monitor the accumulation of coprostanol and the LABs in three tissue pools (digestive gland, gonadal/mantle tissue, and residual tissue) of *Mytilus* exposed to sewage-contaminated waters. The rationale for choosing these three tissues was to isolate potential contributions by ingested particles in the digestive gland, determine if changes in the gonadal lipid content affected tissue concentrations in any of the three tissue pools, and allow the summation of these tissue concentrations in order to compare these data with whole organism composites. We report here preliminary results of a transplant study conducted during the summer of 1988, in which the tissue distributions of these compounds and bioaccumulation rates of the LABs have been determined. The results of the transplant study are compared with time series data of an indigenous population of mussels at the same site.

EXPERIMENTAL

Mussel Deployment and Collection

Mussel specimens were collected from below mean low water at a reference location (Sandwich, MA). This is a nonurban site, and previous analyses of mussels from this population have indicated low levels of anthropogenic organic compounds.[35] Results of the time zero marker analyses, reported below, confirm these organisms to be unimpacted by domestic wastes. The samples were stored over ice and returned to the laboratory. In the lab, mussels were cleaned of epiphytes, and specimens between 3 and 7 cm in length were randomly allocated to 16 polyethylene cages (30 to a cage). The cages were placed in recirculating seawater overnight, and the following day 15 cages were transported (over ice) to a location about 100 m from one of the Nut Island wastewater outfalls (Figure 9.2). Nut Island is one of two municipal wastewater treatment plants discharging into Boston Harbor. The cages were sus-

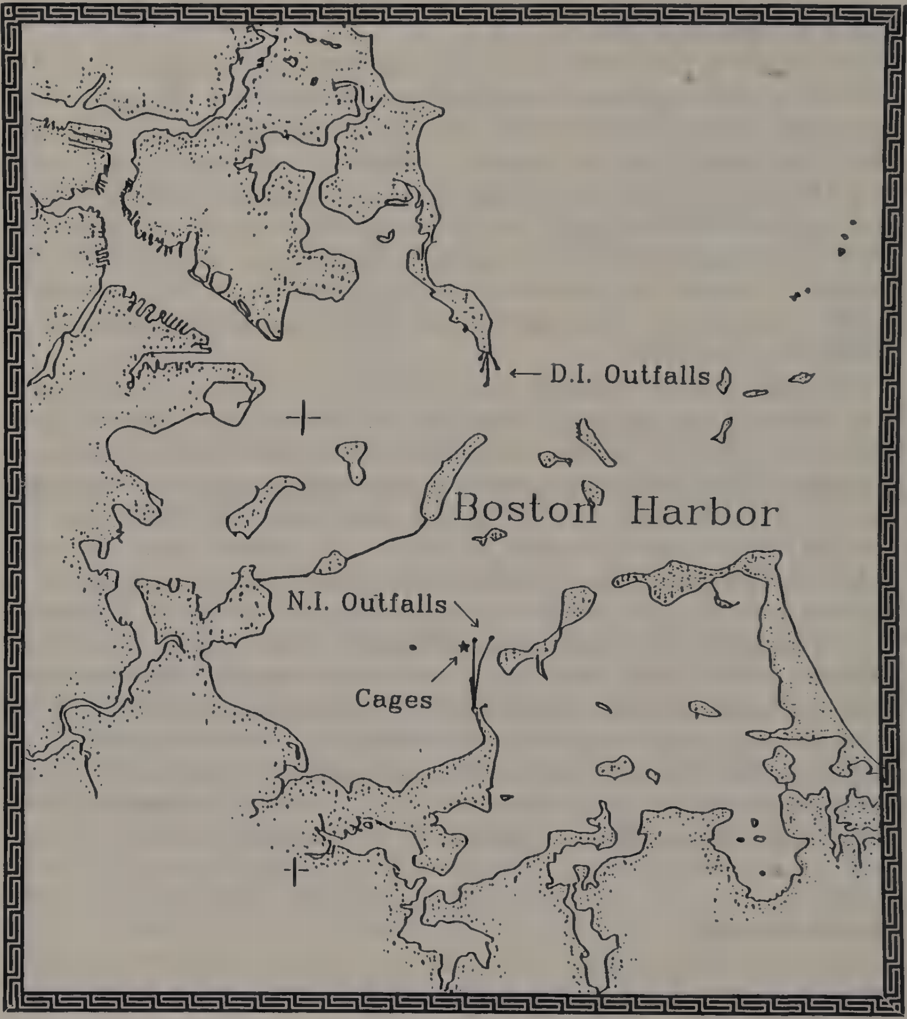

Figure 9.2. Map of Boston Harbor showing location of the Nut Island wastewater outfalls and the navigational marker where the mussel deployment was located.

pended so that they were always at least a meter below the water surface. At 4-week intervals the organisms were transferred to clean cages due to fouling. At these times the organisms' shells were also cleaned.

Three cages were retrieved on each of the following dates: July 25, August 6, September 2, and September 30, 1988. Native specimens growing on a navigational marker at the site were also collected at these times (Figure 9.2). The mussels were cleaned of epiphytes and stored over ice until dissection. All organisms were dissected within 72 hr of collection. Fifteen organisms were randomly selected from each of the three cages per date. Specific tissue types (digestive gland, gonadal-mantle tissue, residual tissues) from these 15 organisms per cage, were pooled to form three replicate (5 specimens per composite) samples for each tissue type, on each date. The residual tissue was composed of those soft tissues remaining after removal of the digestive gland and gonadal/mantle tissue. It consisted of the foot, gills, muscle, and other tissues. The data for the three tissue pools can be combined to yield a value equivalent to a whole organism composite. During dissection, wet weights of the individual tissues were measured, as was shell length, for each organism.

To define whether the reproductive status of the sampled organisms affected the tissue burden of the molecular markers, quantities of indigenous mussels were collected on the last sampling date (September 30). These were dissected, and the few organisms that represented one or the other extreme reproductive state were composited into samples, which will be referred to as "gonadal-rich" and "gonadal-deplete." Since the goal of this exercise was to focus on the status and tissue burden of the mantle/gonadal tissues, the digestive and residual tissues for these samples were pooled during dissection. Composited tissues were stored frozen (–10°C) until analysis could be completed (12 months).

Extraction and Chromatographic Separation

Prior to extraction, the tissue composites (2.5 to 37 g wet weight) were allowed to thaw and transferred in toto to centrifuge bottles containing precombusted anhydrous sodium sulfate, and appropriate recovery surrogates (androstanol, *n*-nonylbenzene, *n*-decylbenzene, *n*-undecylbenzene, *n*-dodecylbenzene, *n*-tridecylbenzene, *n*-tetradecylbenzene) were added. This mixture of tissue, sodium sulfate, and solvent (5–10 mL dichloromethane per gram of wet tissue) was homogenized using a Tekmar Tissumizer. Samples were extracted four times, using the Tissumizer homogenization to facilitate extraction. After each extraction, the mixture was centrifuged (1350 G, 15 min), and the supernatant was decanted and combined with previous extracts of that sample. The extracts were reduced in volume using rotary evaporation. Gravimetric analyses of lipid concentration were performed on aliquots (3 to 7 measurements, 3 to 9 μL each) of the concentrated dichloromethane-lipid extract using a Cahn 29 microbalance. The mean of all measurements for a given extract was used to calculate the total amount of lipid extracted. It should be noted that different extraction methods will result in different lipid yields.[36,37] Thus, the lipid results presented here may not be directly comparable to studies in which different extraction techniques have been used.

An aliquot corresponding to approximately 20 mg lipid was applied to a 1.0 × 30-cm column packed with alumina over silica gel (1:2 v/v, each deactivated 5% with water).[19,38] Four fractions were eluted from this column using hexane (10 mL × 2 fractions), 5% and 30% dichloromethane in hexane (20 mL and 40 mL, combined), and ethyl acetate (40 mL) in succession. The LABs elute with the second fraction, and the sterols in the fourth. The other fractions were not analyzed. Further details of the analytical and separation procedures are reported elsewhere.[19] The sterol fraction was acetylated prior to analysis via gas chromatography using anhydrous pyridine and acetic anhydride.[39] The LAB fraction was concentrated under a stream of dry nitrogen just prior to GC analysis.

Rather than presenting the bioaccumulation results for each of the 26 LABs, the data are given as a summation of all measured LABs. Prior to summing the concentrations of the individual LABs, we evaluated whether they showed any effects of degradation or weathering (i.e., showed variations in isomeric composition). The ratio of internally to externally substituted isomers of the 12 carbon alkyl chain length, which has been suggested to indicate the extent of degradation of the LABs,[17] was determined for all samples. There were no consistent differences in this ratio between tissue pools studied, or over time. This would suggest that degradation was not a cause of differences in LAB concentrations.[19] Summation of the individual LAB isomers provides a convenient means of presenting and comparing the preliminary bioaccumulation results, without an unacceptable loss of information regarding one potential cause of observed differences (i.e., degradation).

Instrumental Analysis

A Varian 6000 gas chromatograph equipped with a hydrogen flame ionization detector and a splitless injector of the design described by Grob[40] was used for quantitation. Analytical separations were performed using 30-m fused silica capillary columns (0.25 mm i.d., film thickness of 0.25 μm). The stationary phases were DB-5 (LABs, J. and W. Scientific) and 50% methyl, 50% phenyl silicone (sterols, Quadrex OV-17 equivalent). The oven temperature was held at 45°C for injection, and the columns were temperature programmed as follows:

- LABs: isothermal at 45°C for 5 min and temperature programmed at 6°C/min to 285°C, with a 30-min hold
- sterols: held at 45°C for 1 min, run up to 255°C at 10°C/min, 2°C/min to 265°C, 1°C/min to 285°C, where it was held for 30 min

Chromatographic data were acquired and integrated using a Nelson Analytical 3000 chromatography data system equipped with a Nelson 763SB intelligent interface. The analytes were quantitated by the internal standard method (*n*-pentadecylbenzene and cholestane as quantitation standards). The detector response was calibrated daily. Recoveries of surrogates (mean ± 1 standard deviation) were *n*-nonylbenzene, 70.51% ± 15.52; *n*-decylbenzene, 91.67% ± 19.54; *n*-undecylbenzene, 98.53% ± 23.60; *n*-dodecylbenzene, 97.37% ± 16.56; *n*-tridecylbenzene, 93.70% ± 15.32; *n*-tetradecylbenzene, 98.47% ± 29.74; and androstanol, 97.97% ± 13.45. Some of the *n*-phenylalkanes used had occasional interferences with coeluting peaks. The absence of similar interference with the secondary phenylalkanes in these samples was confirmed using mass spectroscopy. Because of the generally high recoveries, and the

Table 9.1. Mean Shell Lengths and Tissue Wet Weights of the Composited Mussel Samples of This Study

		Mean Tissue Weight (g) (± std. dev.)[a]		
Date	**Shell Length (cm)**	**Digest.**	**Gonadal**	**Residual**
Indigenous organism samples				
3/23/88	6.32 ± 0.54	8.20	18.95	33.41
5/ 5/88	5.42 ± 0.62	4.72	10.93	20.70
7/ 8/88	4.23 ± 0.60	3.00 ± 0.23	3.77 ± 0.02	13.03 ± 0.08
7/25/88	4.88 ± 0.52	2.64 ± 0.19	3.58 ± 0.06	13.54 ± 1.58
8/ 6/88	5.49 ± 0.50	4.09 ± 0.28	6.39 ± 1.61	21.59 ± 1.50
9/ 2/88	5.39 ± 0.44	4.08 ± 0.42	5.39 ± 0.26	19.82 ± 1.22
9/30/88	6.05 ± 0.51	5.08 ± 0.05	11.96 ± 4.45	26.03 ± 4.00
Transplanted organism samples				
7/ 8/88	6.23 ± 0.83	5.22 ± 1.36	10.54 ± 2.00	24.85 ± 3.96
7/25/88	6.41 ± 0.54	5.68 ± 0.76	12.81 ± 1.73	27.95 ± 4.67
8/ 6/88	6.33 ± 0.44	6.36 ± 0.59	12.17 ± 2.36	28.96 ± 1.79
9/ 2/88	6.44 ± 0.48	5.33 ± 0.29	14.19 ± 4.47	30.20 ± 2.87
9/30/88	6.25 ± 0.56	5.77 ± 0.46	16.50 ± 3.74	33.07 ± 3.74

[a] Total composited tissue weights of the three tissue pools, with standard deviation between replicates of tissue pools on a given date.

sporadic interference with some of the surrogates, none of the data presented here have been adjusted for recovery. An estimate of the precision of the analytical procedure was made by analyzing a whole organism composite that had been homogenized using a Vertis Blender. Subsamples of this tissue mixture were taken and extracted using the procedure described above. The total LAB concentrations, determined on a lipid-normalized basis, for three subsamples of this mixture had a coefficient of variation of 12.4%.

RESULTS AND DISCUSSION

Wet and Lipid Weights

Potential biological effects that might affect the measured tissue burdens of the marker compounds were evaluated by monitoring organism size, tissue wet weights, and lipid content over the course of the study. For comparison, data from two indigenous organism samples collected earlier in 1988 are also presented (March 23 and May 5). The shell length data (Table 9.1) indicate that the mean size of the transplanted mussel samples did not change during this study, whereas the size of the indigenous organisms did. These latter samples had a minimum size (4.23 ± 0.6 cm) at the beginning of the transplant experiment (July 8). Organisms collected subsequently were of larger size, reaching a maximum size (6.05 ± 0.5 cm) on the September 30 sampling date, when the sizes sampled for the two populations overlapped. The changes in shell size of indigenous organisms may indicate that different age classes of these organisms were collected and analyzed. Differences in tissue burdens between the two populations, and over time for the indigenous population, could result from different periods of exposure to the effluent. This would affect tissue burdens if the compounds were accumulated without elimination (i.e., if tissue concentrations result from cumulative exposure and do not represent equilibrium of the tissues with their environment).

The wet weights for both the indigenous (two replicates except March 23 and May 5) and transplanted (three replicates) composited tissue samples (Table 9.1) roughly follow the changes in shell length. The transplanted organisms appear to show no change, or a slight increase, in the wet weights of the tissue type composites over the course of this study (Table 9.1). Since there are only small variations in the wet weights between times, any differences in the tissue burden of the marker compounds in the transplanted mussels are probably not a result of changes in the size or wet tissue weights of organisms being sampled. However, the changes in size and wet tissue weights for the native mussels taken during this study could possibly affect the concentrations of the marker compounds of these samples for the reasons discussed above.

The concentrations of extractable lipid (milligrams lipid per gram wet weight) determined for each of the mussel tissue pools are presented in Figure 9.3. Fluctuations in lipid concentration (Figure 9.3) of the tissues generally follow those of wet weight and shell size. Lipid content is highest in the digestive gland, with the gonadal/mantle tissues exhibiting slightly higher concentrations than those found in the residual tissues, at most times. The lipid content of the digestive gland in the indigenous population is generally greater than that of the transplanted organisms; the lipid content of the other tissues for these two populations are more similar. One possible explanation of the observed differences in lipid content of the digestive gland could be a physiological response to (assumed) environmental stress for the indigenous population. Another would be diversity between the two mussel populations.

Changes in the reproductive status of a mussel population could be indicated by enrichment of the lipid content of gonadal tissues (reflecting increases in gametogenic tissues). If the whole population undergoes a spawning event, the amount of lipid available for contaminants to partition into, as well as the lipid concentration (mg lipid/g wet weight), should undergo dramatic change. No such changes were observed in the lipid content of the gonadal/mantle tissue or in the other tissue pools sampled from either population (Figure 9.3). This suggests that there was no point in time where a majority of either of these populations were involved in spawning. These results agree with subjective observations of the mussel tissues during dissection, and with histological measurements made of tissues from other mussel populations in Boston Harbor.[41] These mussel populations seem to spawn continuously throughout the summer (i.e., there is always a portion of the population involved in spawning), rather than simultaneously spawning once or only a few times during the summer.[41] This reproductive behavior apparently results in the tissue lipid values remaining fairly constant in these samples during the course of this study.

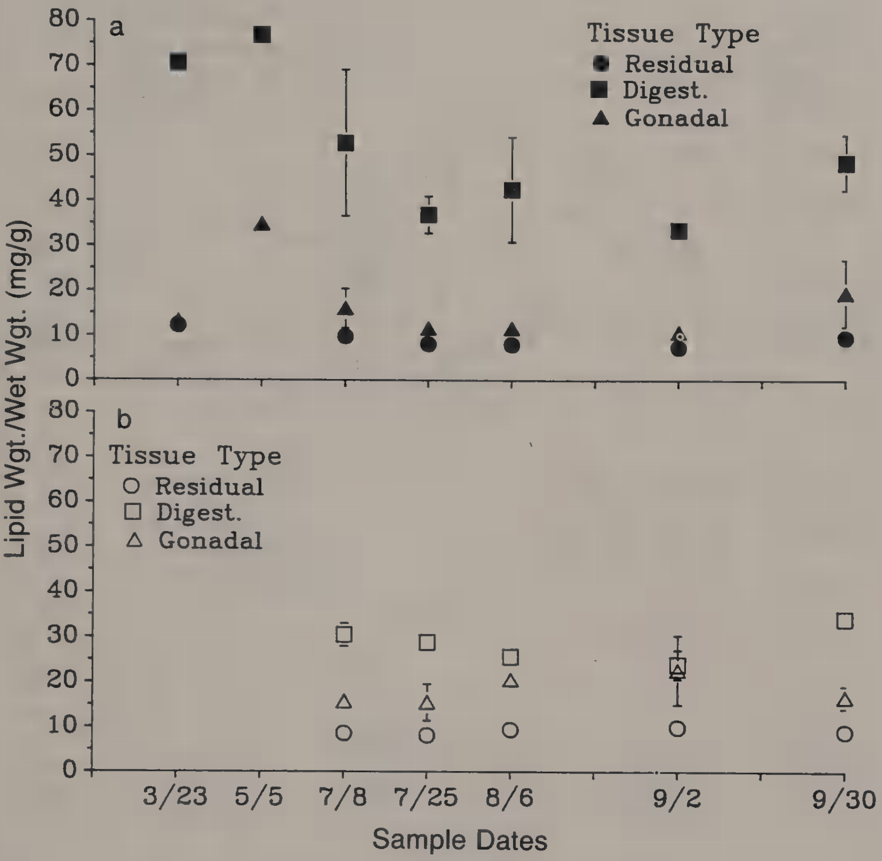

Figure 9.3. Mean (± one standard deviation) lipid concentrations of the tissue pool composites (mg lipid/g wet tissue): *residual* = residual tissues, *digest.* = digestive gland, *gonadal* = gonadal/mantle tissues: *(a)* indigenous organisms, *(b)* transplanted organisms.

Bioaccumulation of Molecular Markers

Tissue concentrations of the molecular markers are reported normalized to lipid weight (Figures 9.4 and 9.5). Coprostanol concentrations in tissues of the indigenous organisms (Figure 9.4a) are quite variable, with no consistent difference between tissue types. The transplanted organisms (Figure 9.4b) show low initial coprostanol concentrations and steady accumulation in the residual and gonadal/mantle tissues. The time zero transplant samples had undetectable amounts of coprostanol in the digestive and residual tissue samples, whereas one of the three gonadal/mantle tissue extracts had a chromatographic peak with the retention characteristics of coprostanol; however, mass spectroscopy could not confirm its identity (other than to indicate a sterol structure).

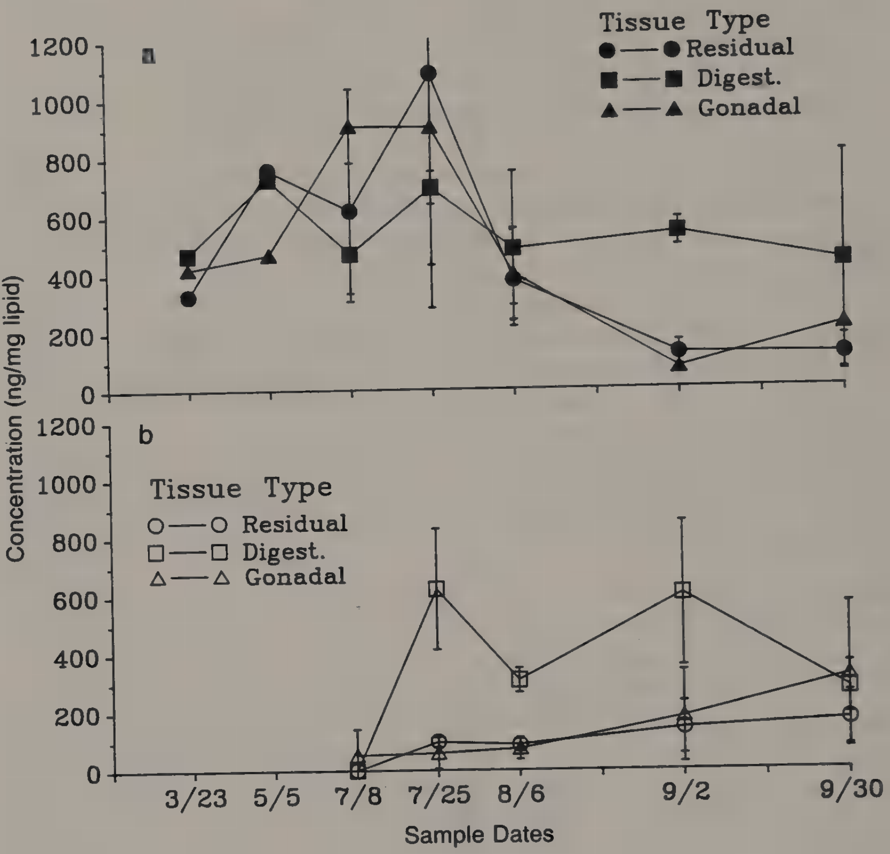

Figure 9.4. Mean (± one standard deviation) coprostanol concentrations in tissue pools (ng coprostanol/mg lipid); symbols as in Figure 9.3.

Coprostanol concentrations in the transplanted organisms were higher in the digestive gland than in the other tissues through most of the study. In both populations the coprostanol concentrations in the gonadal/mantle tissues and the residual tissues are usually similar. On the last two sample dates of this study, the indigenous sterol distributions seem to be more stable. At these times the gonadal/mantle and residual tissues show similar concentrations between the two populations as well.

Jarzebski and Wenne investigated the tissue distributions of sterols in *Macoma balthica* and suggested that the digestive gland may be a sterol storage site, with mobilization to gonadal tissue during gametogenesis.[42] If this also occurs in *Mytilus*, the scatter in native organism coprostanol tissue concentrations may reflect varying contributions to the composite samples from individuals at different stages of reproductive development. This could indi-

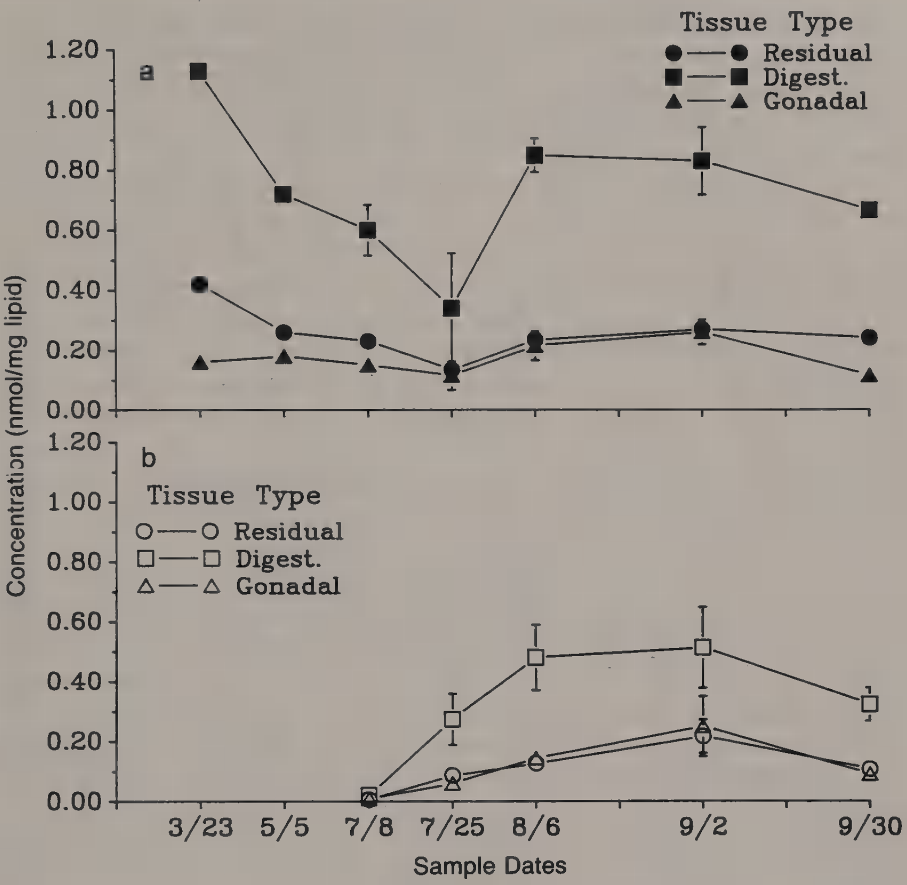

Figure 9.5. Mean (± one standard deviation) of total LAB concentrations in tissue pools (nmol ΣLABs/mg lipid); symbols as in Figure 9.3.

cate that once present in the tissues, coprostanol can be regulated by the organism's normal sterol metabolism.

Tissue distributions of the total LABs (Figure 9.5) are qualitatively similar to those of coprostanol. In both mussel populations the gonadal/mantle and residual tissue pools have similar tissue concentrations. Also, while the transplanted organisms had undetectable concentrations of LABs in all three tissue pools at time zero, by the eighth week the two populations exhibit similar LAB concentrations for residual and gonadal/mantle tissue pools. Total LAB concentrations in the digestive glands are usually higher on a lipid basis than concentrations found in the other tissues. This is consistent with previous reports, which have shown this tissue to be an important storage site for accumulated hydrocarbons.[43-45] For example, Lee et al. reported rapid accumulation of hydrocarbons (alkanes, aromatics) from seawater solutions.[43]

They found these hydrocarbons to be initially taken up by gill tissues, with longer-term accumulation in the digestive gland. Widdows et al. also report higher levels of hydrocarbon accumulation in the digestive gland than by other tissues.[44,45] Further, the digestive gland had higher concentrations of lipids than the other tissues studied.[44]

The elevated concentrations of LABs determined in the digestive glands could reflect contributions of ingested particles, greater partitioning because of the larger lipid pool, or different routes of bioaccumulation for the various tissues. When mussels were exposed to "the water accommodated fraction" of North Sea oil with or without food particles present, organisms exposed to oil with food particles had higher concentrations of aromatic hydrocarbons in all tissues than did organisms exposed to food and oil separately.[44] These differences were most likely due to adsorption of the aromatics to the food particles with subsequent accumulation.[44] This suggests that ingestion of contaminated food particles may be an important route of exposure and bioaccumulation.

The concentrations of the molecular markers in the digestive gland, however, are unlikely to result solely from contributions of sewage-derived particles. Water column particles collected and analyzed during this study had total LAB and coprostanol concentrations in the range of 120 to 595 ng/mg lipid and 670 to 6400 ng/mg lipid, respectively.[19] The digestive gland tissues reached concentrations of 125 to 275 ng/mg lipid (total LABs) and 400 to 700 ng/mg lipid (coprostanol). Using the LAB/lipid concentration to estimate the amount of lipid in the digestive gland that could represent ingested particles indicates that between 20 and 100% of the lipid in this tissue would need to be from ingested particles. Since the lipid content of transplanted mussels digestive gland samples remained fairly constant over the course of this study (Figure 9.3), the elevated LAB concentrations in the digestive gland are unlikely to result solely from the presence of sewage particles.

The attempt to identify native organisms believed to represent extremes of the reproductive cycle, on the last sampling date (September 30), yielded only enough specimens for three samples (two "gonadal-rich" and one "gonadal-deplete"). The concentrations of the molecular markers in these tissue pools (Table 9.2) are compared with other indigenous samples collected on this date. Even though the concentration of lipid in the "deplete" tissues is less than in the other samples, the marker concentrations in the "rich" and "deplete" samples are similar when normalized to lipid weight (Table 9.2). This indicates that while total lipid concentration may affect the mass of an analyte accumulated in these tissues, the lipid-normalized marker concentrations are less dependent on reproductive state. Coprostanol concentrations for these tissues show no relationship with total lipid. The residual plus digestive tissue pools of organisms at the two extremes of reproductive status have similar concentrations, which are higher than those found in the other native organisms sampled on this date. The coprostanol concentration of the gonadal/mantle tissue of the "deplete" sample is roughly the same as that determined for the other

Table 9.2. Values for Lipid Normalized to Wet Weight and the Molecular Marker Concentrations in "Gonadal-Rich" and "Gonadal-Deplete" Tissue Samples

Sample Name	n	Lipid/Wet Weight (mg/g)		Coprostanol (ng/mg)		LABs (nmole/mg)	
		Mean	Std. Dev.	Mean	Std. Dev.	Mean	Std. Dev.
"Gonadal-Rich" (12 weeks)							
Resid./Digest.	2	17.00	0.80	415.87	131.54	0.47	0.08
Gonadal/Mantle	2	19.75	4.74	302.29	58.34	0.22	0.01
"Gonadal-Deplete" (12 weeks)							
Resid./Digest.	1	10.41	—	463.55	—	0.48	—
Gonadal/Mantle	1	8.82	—	209.13	—	0.29	—
Indigenous (12 weeks)							
Resid./Digest.	2	16.08	1.30	257.11	190.97	0.45	0.03
Gonadal/Mantle	2	19.55	7.53	213.03	0.59	0.14	0.05

Note: n indicates the number of samples averaged to give the results listed.

Table 9.3. Results of the ANOVA Analysis of Total LAB Concentration in the Three Tissue Types over Time

Source of Variation	Sum of Squares	DF	Mean Square	F	Significance of F
Total	92.8	35	2.7		
Explained	83.5	11	7.6	19.6	≤0.001
Main effects	80.2	5	16.0	41.4	≤0.001
Time	21.1	3	7.0	18.2	≤0.001
Tissue type[a]	59.0	2	29.5	76.1	≤0.001
1 vs 2 + 3	59.0	1	59.0	171.1	≤0.001
2 vs 3	0.002	1	0.002	0.01	0.93
2-Way interactions					
Time with tissue type	3.3	6	0.6	1.4	0.25
Time with (1 vs 2 + 3)	2.9	3	1.0	2.8	0.06
Time with (2 vs 3)	0.4	3	0.1	0.6	0.65
Residual	9.3	24	0.4		

[a] Tissue types are 1 = digestive gland, 2 = gonadal/mantle tissue, 3 = residual tissues.

native samples, though it is lower than the concentration of the "rich" gonadal/mantle tissues.

Differences between tissue types (gonadal/mantle versus digestive and residual) are also evident for the LABs. The LAB concentration of the residual and digestive tissue pool is roughly twice that of the gonadal/mantle tissues. The effect of tissue type on LAB concentration indicates that different processes may be controlling the contaminant burdens of the various tissues. This data set is inadequate to assess what those processes might be, though they may include route of exposure and elimination.

Bioaccumulation of the LABs

The differences in total LAB concentrations of the three tissue types over time were subjected to analysis of variance (ANOVA). The variables considered were LAB concentration, time, and tissue type, with time being treated as a "fixed effect" (model I ANOVA; time zero samples excluded from this analysis because their zero variance caused a violation of the assumption of homogeneity of variance).[46] The results of the ANOVA analysis are shown in Table 9.3. This table shows the total, explained, and residual variance for this analysis. The explained variance is broken down into that explained by LAB concentration over time, and that explained by tissue type. The variance explained by tissue type is further defined by making comparisons among tissue types. As there are only two degrees of freedom, only two comparisons could be made. Since the LAB concentration in the digestive gland could potentially reflect contributions by particle-associated LABs, it was decided to compare the concentration of this tissue with that of the other two tissue types. This comparison was made by combining the results for the gonadal/mantle and residual tissues and contrasting their tissue burdens with that of the digestive gland. The remaining comparison was then applied to the LAB concentrations of the gonadal/mantle tissue and the residual tissues.

Interaction between the variables would decrease the utility of the ANOVA analysis. The results (Table 9.3) show insignificant interaction between time and tissue type, as well as between time and the gonadal/mantle and residual tissue comparison. The interaction term for the comparison between the digestive gland and the other tissues is only marginally insignificant ($\alpha = 0.056$); a reason for this potential interaction would be that the LAB concentration change over time, in these tissues, is quite different (i.e., the slopes of the tissue concentrations over time are not parallel). This is in contrast to the changes in LAB concentrations over time for the residual and gonadal/mantle tissues, which are very similar.

Since the main effects show insignificant interaction (except as noted), we can utilize the ANOVA analysis to evaluate the LAB bioaccumulation in the tissues over time. The analysis shows that there is a significant time effect for the LAB tissue concentrations, which when evaluated with Figure 9.5, can be considered to be indicative of their bioaccumulation. There is also a significant tissue type effect on the LAB concentration. The comparisons between tissue types shows that there is a significant difference between the LAB concentrations of the digestive gland and the (pooled) data for the other tissues, while there is an insignificant difference in the LAB concentrations of the gonadal/mantle and residual tissues. In summary, there is significant bioaccumulation of the LABs by these tissues over time. The LAB concentrations in the digestive gland are significantly greater than those of the other tissues. The difference between the LAB concentrations of the gonadal/mantle tissue and the residual tissues is insignificant.

Bioaccumulation Rate of the LABs

As noted earlier, it would be useful to estimate the bioaccumulation rate of these compounds prior to using them to indicate the contribution of a municipal wastewater effluent to the tissue burden of other nonspecific pollutants. The accumulation rate of the LABs in mussel tissues over the first 4 weeks of this study (i.e., days 0 to 29) has been estimated. This time period was chosen because there was nearly linear accumulation over this period, suggesting elimination (depuration) could be ignored. The assumption that elimination is negligible allows the bioaccumulation rate to be derived using linear regression of the tissue concentrations versus time. The accumulation rates for the different tissues were calculated using the first-order rate equation:[47]

$$\ln C_2(t) = k_1 t + \ln (C_2(t=0)) \qquad (9.1)$$

where C_2 = tissue concentration (nmol/mg lipid) of total LABs
t = time (days)
k_1 = bioaccumulation rate (nmol/mg lipid^{-1}/day)

Table 9.4. Estimated Bioaccumulation Rates of Total LABs in the Transplanted Organisms in Each Tissue Type over the First Four Weeks of the Transplant

	$\ln C_2 (t) = k_1 t + \ln (C_2 (0))$	n	R^2
Digestive Gland	$\ln C_2 (t) = 0.14 (\pm 0.04) - 4.44 (\pm 1.29)$	9	0.69
Residual Tissues	$\ln C_2 (t) = 0.12 (\pm 0.03) - 5.21 (\pm 0.93)$	9	0.76
Gonadal/Mantle Tissue	$\ln C_2 (t) = 0.08 (\pm 0.01) - 4.40 (\pm 0.38)$	8	0.89

Note: As in Table 9.2, n refers to the number of data points used to generate the regression line.

The resulting lines for the three tissues are shown in Table 9.4. The slopes (accumulation rate) for the three tissues vary. The digestive gland has the highest rate, and the gonadal/mantle tissue the lowest. The bioaccumulation rates again indicate that the digestive gland is one of the more important tissues for LAB accumulation.

CONCLUSIONS

This work has shown that bioaccumulation of these molecular markers by *Mytilus edulis* is rapid (i.e., 2–4 weeks). Further research should indicate whether these compounds mimic other hydrophobic contaminants with regards to transport, availability, and accumulation.

The data presented show that by the eighth week, the two populations had similar tissue distributions of the molecular markers. The marker concentrations and tissue distributions of the indigenous population indicate that the differences in the size and wet weight of organisms collected had little effect on the tissue burdens observed. The potential that coprostanol tissue concentrations may be regulated by the mussel's normal sterol metabolism complicates the interpretation of tissue data for this compound and may limit its usefulness as a molecular marker.

The digestive gland of *Mytilus* is a lipid-rich tissue, which also seems to function as a storage tissue. The apparent reproductive state of the organisms, and the total amount of (reproductive) lipid available, may have little bearing on tissue concentrations when these are normalized to the tissue lipid concentration (e.g., ng marker/mg lipid). There were significant differences between the LAB concentrations of the digestive gland and the other tissues, even when normalized to lipid content. This may indicate that there are different processes contributing to the contaminant burden of the various tissues for these organisms. These differences could be related to the digestive gland functioning as a storage tissue for the organism.

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CHAPTER 10

Bioaccumulation of *p,p′*-DDE and PCB 1254 by a Flatfish Bioindicator from Highly Contaminated Marine Sediments of Southern California

David R. Young, Alan J. Mearns, and Richard W. Gossett

INTRODUCTION

Bottom sediments are a major reservoir for residues of the pesticide DDT and polychlorinated biphenyls (PCBs) released into aquatic environments. Fish consumption warnings or fishery closures in areas polluted by these chlorinated hydrocarbons are increasing. Thus, it is important to understand the processes by which such hydrophobic neutral synthetic organic compounds are incorporated into tissues of benthic seafood organisms. The fugacity model of bioaccumulation states that uptake is determined by the chemical fugacity differential between the organism and its environment. For benthic species this model most conveniently is tested by measuring residue concentrations in tissue (C[t]) and in the sediment (C[s]) to which the organism has been exposed. Here we describe such a test conducted through a field study of surficial sediments and a flatfish used successfully as a bioindicator for chlorinated hydrocarbon contamination in the Southern California Bight.

BACKGROUND

Numerous investigations over the last two decades showed that concentrations of DDT and PCB residues in sediments and organisms from the Southern California Bight were among the greatest reported for any coastal marine ecosystems.[1-10] The principal constituents of these residues have been identified, respectively, as *p,p′*-DDE and a PCB mixture most closely resembling Aroclor 1254.[11-12] Highest values occurred on the Palos Verdes Shelf, which received municipal wastewater discharges from the Joint Water Pollution Control Plant (JWPCP) submarine outfall system of the Los Angeles County Sanitation Districts. Bottom sediments on the shelf also contained relatively high concentrations of organic material and supported large populations of certain benthic/epibenthic organisms, such as the Dover sole (*Microstomus pacificus*). During the 1970s this flatfish, which often is found partially buried in the surficial sediment layer, was severely affected by a fin erosion disease.[13] Distinct gradients of both the incidence of this disease[14] and tissue concentrations of DDTs and PCBs,[15] generally associated with the sediment contamination gradient away from the JWPCP outfall system, suggested that this flatfish was a potentially useful bioindicator of benthic pollution in the Southern California Bight.[16] Therefore, when an extensive survey of bottom sediments was conducted during 1977 along the 60-m isobath of the Southern California coast,[17-18] tissues from Dover sole specimens also were collected and analyzed from a number of sites both on the Palos Verdes Shelf, and from reference zones to the north and south of this highly contaminated area.

PROCEDURES

A synoptic collection of surficial bottom sediments was obtained during summer 1977 with a modified van Veen grab sampler from a water depth of 60 m at numerous stations along the southern California coast.[17-18] The positions (latitude and longitude) of these stations are listed by Word and Mearns.[18] A single grab sample was taken at each station, and the upper 2 cm was subsampled using a clean stainless-steel spatula. Specimens of the Dover sole were collected in bottom trawls conducted along transects near five of the sediment stations on the Palos Verdes Shelf (JWPCP Monitoring Program trawl transects T1–200′ through T5–200′), and near ten stations in the reference zone to the north and south of the shelf (Figure 10.1). In three cases, the trawls were made between sediment stations in the reference zone. Therefore, average values for each pair of sediment samples (from stations 19 and 21; 41 and 45; 45 and 49) were taken as estimates of the surficial sediment concentrations to which flatfish from these trawls were exposed. Generally, six specimens were taken from each station trawl for analysis. The samples of sediment (in precleaned glass bottles with Teflon-lined caps) and flatfish (in plastic bags) were returned to the laboratory and frozen on the day collected, pending processing for analysis. Using procedures described in Word and Mearns,[18] aliquots of the homogenized sediment samples were analyzed for several conventional sediment parameters including total volatile solids (TVS). The methods described by Young et al. were used for the analysis of *p,p′*-DDE and PCB 1254.[5,11] First, approximately 40 g of wet sediment were oven-dried at 60°C for 24 hr. The sample then was extracted with *n*-hexane and cleaned up on activated Florisil; one-half of the extract was saponified for PCB analysis. Measurements on these extracts were conducted as described below.

Using a metal scalpel with a carbon-steel blade, the flatfish specimens were dissected while still semifrozen to minimize contamination of the muscle and liver tissue samples by mucous or visceral fluids. Approximately 5–10 g of wet muscle and the entire liver (typically weighing 1–5 g) were taken for DDE and

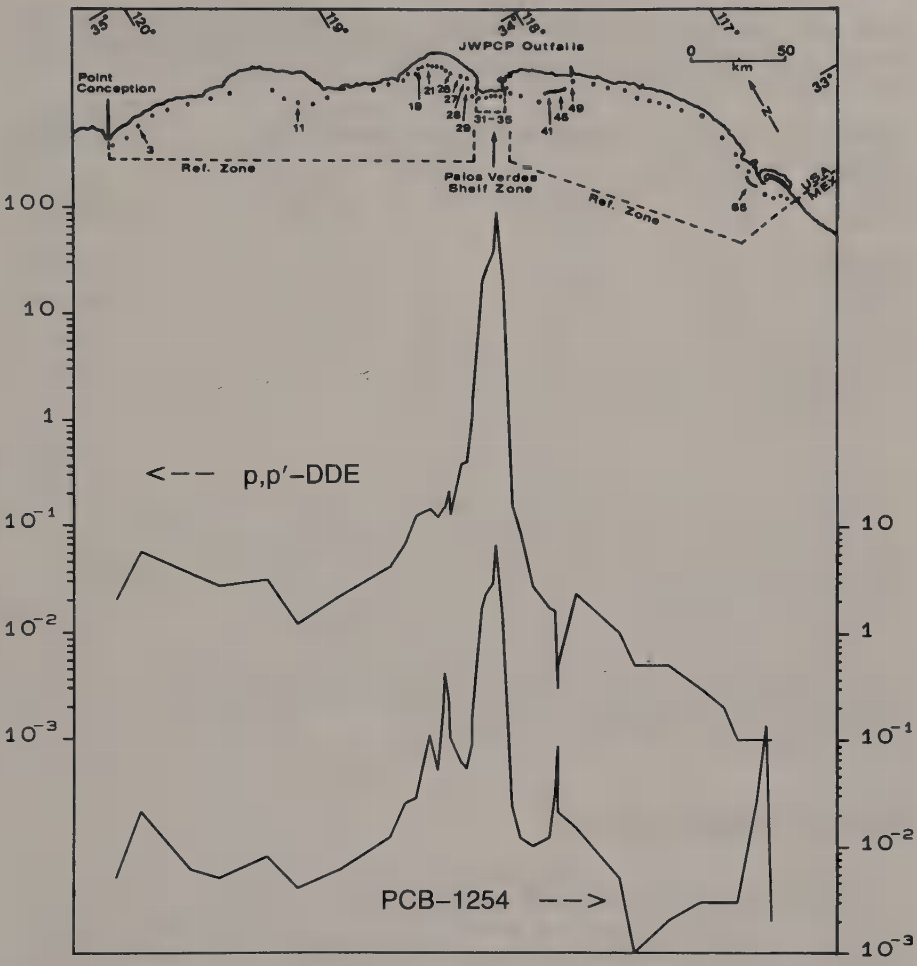

Figure 10.1. Southern California Bight station locations for the 1977 60-m surficial sediment collections, and corresponding concentrations (mg/kg dry wt.) of *p,p′*-DDE and PCB 1254. Station numbers for sites of Word and Mearns[18] included in this study, and associated reference and Palos Verdes Shelf zones, are indicated.

PCB analysis. In addition, aliquots of these tissue samples obtained from the six specimens collected at two of the Palos Verdes Shelf stations (33 and 34) and at three reference zone stations (41/45, 45/49, and 65) were analyzed for lipid content according to the chloroform/methanol extraction procedure of Bligh and Dyer.[19]

Tissue samples to be analyzed for *p,p′*-DDE and PCB 1254 residues were extracted successively in acetonitrile and *n*-hexane. These extracts then were reduced in volume and cleaned up on activated Florisil. Analysis was conducted by electron capture gas chromatography using packed columns (1.5% OV-17 and 1.95% QF-1 on Gas Chrom Q). Quantitation of *p,p′*-DDE was

Table 10.1. Median Values (and Their Ratios) for Station Concentrations of Sediment Total Organic Carbon, *p,p′*-DDE, and PCB 1254 in the Palos Verdes Shelf ($n = 5$) and Reference ($n = 10$) Zones

Zone	TOC (% dry wt.)	p,p′-DDE (μg/g dry wt.)	PCB 1254 (μg/g dry wt.)
Shelf:			
Median	7.6	27	2.3
Range	(4.3–12)	(20–92)	(1.4–6.6)
Reference:			
Median	0.62	0.09	0.06
Range	(0.26–3.7)	(0.001–1.1)	(0.004–0.10)
Ratio of Zone Mds.	12	300	38

Note: Sediment total organic carbon calculated from TVS values using the regression:[22]
% TOC = 0.484 (% TVS – 1.86)

accomplished by direct comparison of its peak height with that of a standard obtained from U.S. EPA. The logarithm (base 10) of the octanol:water partition coefficient (log K_{ow}) for this compound is 5.8.[20] PCB 1254 was quantified against a corresponding Aroclor 1254 standard. The major IUPAC congener in the chromatograph profile chosen for this quantitation has been tentatively identified (by coauthor R. W. Gossett) as congener #110, which has a log K_{ow} of approximately 6.5.[21] All sediment and tissue concentrations were corrected for procedural blank and recovery values.

RESULTS AND DISCUSSION

The distributions of *p,p′*-DDE and PCB 1254 in the 1977 collections of surficial sediment from the 60-m isobath of the Southern California Bight are illustrated in Figure 10.1 and are summarized in Table 10.1. The data indicate that median sediment concentrations of these residues on the Palos Verdes Shelf were 38 to 300 times greater than those in the reference zone. In addition, TVS values quantifying organic content of the sediments ranged from 11 to 27% on the shelf, compared to values below 10% in the reference zone. Thus, for the purposes of this analysis the stations were classified into two groups or zones: a high-contamination shelf zone off the Palos Verdes Peninsula (stations 31–35) and a reference zone containing the other ten stations.

Total organic carbon (TOC) was not measured in these sediment samples. However, Mitchell and Schafer obtained the following regression ($r^2 = 0.986$; $p < 0.001$) between surficial sediment concentrations of TVS and TOC in 1974 samples obtained over a zone extending 16 km from the sludge outfall of Los Angeles City's Hyperion Municipal Wastewater Treatment Plant in Santa Monica Bay:[22]

$$\%\ \mathrm{TOC} = 0.484\ (\%\ \mathrm{TVS} - 1.86) \qquad (10.1)$$

Table 10.2. Median Muscle or Liver Tissue Concentrations of *p,p′*-DDE and PCB 1254 for Dover Sole Specimens from Each Shelf Zone Station and Corresponding Overall Median Values (and Ranges of Station Medians) for the Reference Zone

Sediment Station	Trawl Station	Muscle (μg/g wet wt.)			Liver (μg/g wet wt.)		
		n	*p,p′*-DDE	PCB 1254	n	*p,p′*-DDE	PCB 1254
Shelf Zone							
31	T1	2	12	0.51	—	—	—
32	T2	6	16	1.1	6	210	10
33	T3	6	22	1.4	6	240	17
34	T4	6	19	1.2	6	160	12
35	T5	5	8.0	0.21	—	—	—
Median			16	1.1	—	210	12
Reference Zone							
Median			0.24	0.11		0.80	1.5
Range			0.02–2.5	0.01–0.36		0.2–6.7	0.2–5.6
No. Stations			10	10		6	6
Ratio of Zone Mds.			67	10		260	8

Note: Median of individual station median tissue concentrations rounded to two significant figures.

The TVS values used to obtain this regression ranged from 3 to 52%, which encompassed the range (3 to 27%) obtained in the 1977 60-m sediment survey. Thus, this regression equation was used to estimate TOC concentrations from the sediment TVS concentrations.

The sediment concentrations (on a dry weight basis) obtained for the two study zones show that median concentrations of sediment TOC, *p,p′*-DDE, and PCB 1254 for the shelf zone were higher than those for the reference zone by factors of 12, 300, and 38, respectively (Table 10.1). Further, the percent TOC ranges for the two zones (4.3–12 vs 0.26–3.7) did not overlap, and the lower limit of the ppm DDE range (20) for the shelf zone was 18 times the upper limit of the range (1.1) for the reference zone. Similarly, the lower limit of the ppm PCB range (1.4) for the shelf zone was 14 times the upper limit of the range (0.10) for the reference zone. The fact that the surficial sediments of these two zones were so different in these parameters provided a good opportunity to test, from field data, the utility of the fugacity model of benthos:sediment bioaccumulation for a common marine flatfish of the northeastern Pacific.

In this analysis we have elected to use the median tissue concentration as the measure of central tendency for fish muscle or liver contamination at each station. Therefore, in Table 10.2 we list the median concentration (on a wet weight basis) of *p,p′*-DDE or PCB 1254 in Dover sole muscle or liver tissue obtained for individual shelf zone stations. Also shown are the reference zone overall median values (and ranges) obtained from the median specimen concentrations of DDE or PCB for each station in this zone. Liver tissue was analyzed in specimens collected at only three of the five shelf zone stations and six of the ten reference zone stations. The ratios of the zone median values indicate that levels of DDE and PCB contamination in the tissues of specimens from the shelf zone were 67 to 260 and 8 to 10 times greater than those in specimens from the reference zone, respectively.

Previous studies had shown that the lipid content of Dover sole specimens collected from the Palos Verdes Shelf was substantially higher than that measured in specimens collected elsewhere off southern California.[13,23] We obtained similar results. Median (wet weight) muscle tissue concentrations of extractable lipid for the shelf and reference zone specimens analyzed (n = 12 and 18, respectively) were 2.36 and 1.34%; corresponding values for liver tissue from all of these specimens were 24.8 and 13.2%. Thus, our best estimate is that muscle and liver tissue for Dover sole specimens collected during 1977 from the Palos Verdes Shelf each contained approximately 80% more extractable lipid than did corresponding specimens from the reference zone.

The very large zonal differences in sediment and tissue concentrations of DDE and PCB first were used to evaluate the least complex form of the fugacity model.[24] This model is a simple partition coefficient commonly termed the *bioaccumulation factor* (BAF):

$$\mathrm{BAF} = C_t/C_s \tag{10.2}$$

where C_t and C_s are a contaminant's concentrations in the specimen tissue and corresponding sediment samples, respectively. In our approach, median wet-tissue–to–dry-sediment ratios for *p,p′*-DDE and PCB 1254 in specimens from each trawl station were calculated by dividing the station median wet weight tissue concentrations by the corresponding dry weight sediment value (Table 10.3). For consistency of units, bioaccumulation factors typically are obtained from the concentration ratios of tissue and sediment each on a dry weight basis. However, percent moisture values for the flatfish tissues analyzed in this study were not available. Further, the fact that criteria for chlorinated hydrocarbon residues in seafood are promulgated on a wet weight basis, while sediment concentrations typically are reported on a dry weight basis, supports the utility of such a mixed-unit index (modified bioaccumulation factor, MBAF) for evaluating conditions leading to contamination of living resources.

The results listed in Table 10.3 suggest that use of the modified bioaccumulation factor may be misleading regarding the relative bioavailability of the contaminants in the two zones. Whereas the ratio of shelf-to-reference zone median concentrations (wet weight basis) for the four tissue-contaminant pairs ranged from 8 to 260 (Table 10.2), the corresponding ratios for the four modified bioaccumulation factors all were less than 1.0, ranging from 0.07 to 0.20. (We note that, assuming the muscle or liver tissue percent water values are similar for specimens from the two zones, the ratios of zone median BAFs also would be similar to those for the MBAFs given in Table 10.3.) Such MBAF (or BAF) values alone might be interpreted as

Table 10.3. Median *p,p′*-DDE and PCB 1254 Tissue/Sediment Modified Bioaccumulation Factors for Individual Shelf Zone Stations: Ratios of Concentrations in Tissues of Dover Sole Specimens Normalized to Corresponding Surficial Sediment Concentrations and Corresponding Overall Median Values (and Ranges of Station Medians) for the Reference Zone

Sediment Station	Trawl Station	Muscle n	Muscle *p,p′*-DDE	Muscle PCB 1254	Liver n	Liver *p,p′*-DDE	Liver PCB 1254
Shelf Zone							
31	T1	2	0.55	0.30	—	—	—
32	T2	6	0.57	0.50	6	7.7	4.4
33	T3	6	0.57	0.48	6	6.1	5.9
34	T4	6	0.20	0.18	6	1.8	1.8
35	T5	5	0.40	0.15	—	—	—
Median			0.55	0.30		6.1	4.4
Reference Zone							
Median			2.7	2.0		62	59
Range			0.40–32	0.36–6.1		12–410	9–120
No. Stations			10	10		6	6
Ratio of Zone Mds.			0.20	0.15		0.10	0.07

evidence that *p,p′*-DDE and PCB 1254 were less available for accumulation by Dover sole specimens from the Palos Verdes Shelf than by specimens from the reference zone.

Therefore, we examined the next level of the fugacity model. Here the approach utilizes a more complex partition coefficient obtained by normalizing C_t to tissue extractable lipid concentration L, and C_s to sediment TOC concentration.[24] The resultant ratio of normalized concentrations is termed the *accumulation factor* (AF):

$$AF = (C_t/L)/(C_s/TOC) \qquad (10.3)$$

These factors were calculated for *p,p′*-DDE and PCB 1254 concentrations obtained at each trawl station by dividing the station's median concentration for muscle or liver, normalized to the appropriate median lipid concentration value for a given zone and tissue, by the corresponding TOC-normalized sediment concentration (Table 10.4).

This application of the fugacity model of bioaccumulation, incorporating tissue lipid and sediment TOC normalizations, generally yielded good agreement between the degree of contaminant accumulation from bottom sediment (as characterized by station and zone median values) in Dover sole from the two study zones (Table 10.4). Median AF values for *p,p′*-DDE in muscle tissue of specimens from the shelf and reference zones were 1.7 and 1.8, respectively; corresponding AF values for the liver tissue were 2.0 and 3.4. Similar agreement was observed for PCB 1254. Median AF values for the two zones were 0.96 and 1.3 for muscle tissue, and 1.4 and 2.7 for liver tissue. Thus, despite the very large differences in median levels of sediment and tissue contamination between the two zones, this application of the

Table 10.4. Median Accumulation Factors for *p,p′*-DDE and PCB 1254 in Dover Sole Tissues Obtained for Specimens from Each Shelf Zone Station and Corresponding Overall Median Values (and Ranges of Station Medians) for the Reference Zone

Sediment Station	Trawl Station	Muscle			Liver		
		n	*p,p′*-DDE	PCB 1254	n	*p,p′*-DDE	PCB 1254
Shelf Zone							
31	T1	2	1.7	0.96	—	—	—
32	T2	6	1.8	1.6	6	2.3	1.4
33	T3	6	1.9	1.6	6	2.0	1.9
34	T4	6	1.0	0.96	6	0.86	0.91
35	T5	5	0.74	0.27	—	—	—
Median			1.7	0.96		2.0	1.4
Reference Zone							
Median			1.8	1.3		3.4	2.7
Range			(0.38–14)	(0.24–2.3)		(1.2–19)	(1.0–4.2)
No. Stations			10	10		6	6
Ratio of Zone Mds.			0.94	0.74		0.59	0.52

fugacity model of bioaccumulation yielded results for a given tissue and contaminant that each agreed within a factor of two. These results, based on field data obtained from a relatively small number of specimens (≤ 6) per station and of stations (5–10) per study area, indicate the potential usefulness of the approach in evaluating benthic contamination by major DDT and PCB residues on a regional basis.

In addition to providing comparable results for tissue:sediment ratios over a very large range of exposure, application of this fugacity model to the results of our survey yielded AF values that agree with the value predicted from independent laboratory experiments. McFarland[25] and McFarland and Clarke[26] analyzed results of separate experiments on partitioning of hydrophobic neutral trace organics between (1) sediment organic carbon and water and (2) fish and water. Assuming that octanol was a satisfactory surrogate for the total organic carbon pool to which an organism was exposed, they concluded that, under equilibrium conditions, the partition coefficient that is equivalent to the accumulation factor considered here should have a value of about 1.72. The results of our analysis for *p,p′*-DDE and PCB 1254 presented in Table 10.4 are in good agreement with this prediction. The shelf zone median AF value for these two hydrophobic neutral synthetic organics in flatfish muscle and liver range from 0.96 to 2.0, with a median value of 1.55. If the reference zone median AF values are included, the resultant median value is 1.75, similar to the equilibrium value (1.72) predicted by McFarland and Clarke.[26] Further, the lower (0.96) and upper (3.4) limits of the range of eight zonal median AF values agree with the predicted value within a factor of two.

SUMMARY AND CONCLUSIONS

A simple analysis of field-generated data on two hydrophobic (log $K_{ow} \cong 6$) neutral synthetic organic contaminants of the coastal marine ecosystem off southern California supports the fugacity model of bioaccumulation. Limitations of the study that might compromise the accuracy or precision of the results include estimation of sediment TOC from total volatile solids concentrations and extrapolation of tissue lipid median concentrations for each zone to nonanalyzed specimens. Also, the survey design included relatively few sediment ($n = 1$) and flatfish ($n \leq 6$) samples per station, and relatively few stations per study area (shelf zone: $n = 3$–5; reference zone: $n = 6$–10 for liver and muscle tissues, respectively). Further, the variability of sediment exposure experienced by the mobile flatfish specimens trawled near a given sediment station is unknown. Despite these limitations, the accumulation factors obtained for *p,p′*-DDE and PCB 1254 based on lipid normalization of flatfish muscle and liver tissue concentrations, and TOC normalization of surficial sediment concentrations, produced remarkably consistent results. Ratios of zonal median AFs, based on station median AFs for the two tissues and two contaminants, yielded values ranging from 0.52 to 0.94. These results indicated agreement between shelf and reference zone median AF values that was within a factor of two, despite the large range of sediment and tissue concentrations measured in the two study areas.

Finally, the four median AF values obtained for the shelf zone ranged from about 1.0 to 2.0 (median = 1.6). Corresponding results for the reference zone (where concentrations were lower and resultant uncertainties higher) ranged from 1.3 to 3.4 (median = 2.2). The overall median of these eight values was about 1.8, in good agreement with the value of 1.7 predicted for hydrophobic neutral trace organics from analysis of laboratory partitioning experiments. This provides further support for the reliability of the fugacity model of bioaccumulation, and its potential usefulness in predicting levels of such compounds expected in benthic organisms exposed to contaminated bottom sediments.

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PART IV

BIODEGRADATION

A. Anaerobic Dechlorinations

CHAPTER 11

Dechlorinations of Polychlorinated Biphenyls in Sediments of New Bedford Harbor

James L. Lake, Richard J. Pruell, and Frank A. Osterman

INTRODUCTION

The breakdown of polychlorinated biphenyl (PCB) congeners in situ in sediments heavily contaminated with PCBs by processes called *reductive dechlorinations* have been reported.[1-3] These studies characterized several distinct dechlorination patterns, caused by different strains of anaerobic bacteria, which resulted in PCB residues that were altered from the original Aroclor inputs. The upper New Bedford Harbor (NBH), above the Coggeshall St. Bridge (Figure 11.1), is a shallow, approximately 200-acre salt marsh estuary, which received large inputs of Aroclor 1254 (A-1254) and Aroclor 1242 (A-1242) from 1947 to 1970, and possibly Aroclor 1016 (A-1016) from 1970 to 1978, from a capacitor manufacturing plant designated *plant A* in Figure 11.1.[4] Another study found variations in the extent of dechlorination processes in 5- to 7.5-cm and 15- to 17.5-cm sections of cores taken in the northern part of the upper NBH.[5] However, the distributions of PCBs in extracts of sediment core sections taken in the southern part of the upper NBH (Figure 11.1) as part of a pilot dredging study at the Environmental Research Laboratory–Narragansett (ERLN) showed only small alterations relative to mixtures of A-1242 and A-1254. The present study was undertaken to determine the extent of alteration of PCB residues in the sediments of upper NBH resulting from dechlorination processes, and to estimate the rates of these processes.

METHODS

Sediment cores were collected by piston corer in upper and lower NBH during the period July 5–7, 1988, at the locations shown (Figure 11.1). Cores were capped and placed inside sealed plastic bags. The cores were stored on ice shortly after collection and during the transport to the ERLN and then frozen (20°C). From collection to the time they were frozen, cores were held vertically

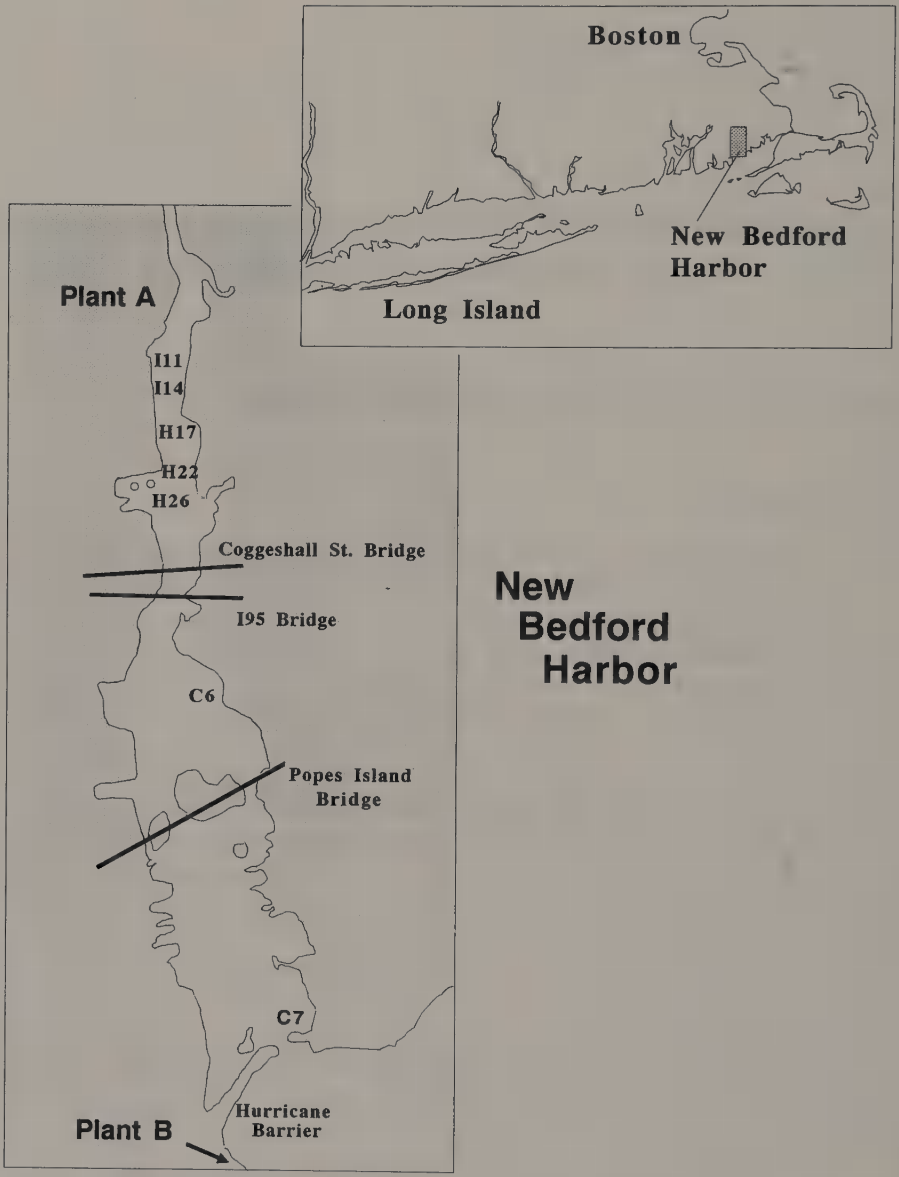
Boston
New Bedford Harbor
Long Island
Plant A
I11
I14
H17
H22
H26
Coggeshall St. Bridge
I95 Bridge
C6
Popes Island Bridge
C7
Hurricane Barrier
Plant B
New Bedford Harbor

Figure 11.1. Map showing locations of electrical capacitor plants *A* and *B* and of cores taken in this study. *Small circles* show locations of sediment samples analyzed in pilot dredging study.

to avoid mixing of sediment layers. Samples of core sections were taken from frozen cores using a power drill and a 2.5-cm diameter hole saw to cut plugs from the cores at the desired depths. The hole saw used for sampling was washed in a soap-and-water solution, followed by washing in acetone between cuttings to avoid cross-contamination of samples. Core sections taken for analysis in this study were obtained at 0–2.5, 5–7.5, 15–17.5, 30–32.5, and 45–47.5 cm, but in shorter cores only the top sections could be sampled. The core section samples were extruded into precleaned glass jars, capped, and stored at −20°C until analysis.

Since this was a field study and examined the consequences of processes that were many years in duration, no true experimental control could be incorporated into the study. Our approach was to compare the distribution of PCBs found in sediments of upper NBH with those found in mixtures of Aroclors that contaminated this area. Additional comparisons of PCB distributions were made between NBH sediments and a nondechlorinated anaerobic sediment from Black Rock Harbor, CT.[6] The latter comparisons documented the extent of changes in PCB distributions in NBH relative to those found in anaerobic sediments where no dechlorination had occurred.

Analytical Methods

Core sections were thawed and mixed thoroughly with a stainless-steel spatula, and a weighed aliquot was dried in a dessicator to a constant weight to determine sediment water content. A 1-g aliquot of the wet sediment was extracted with 5 mL of acetone for 30 sec using an ultrasonic probe (Model W-370, Heat Systems, Ultrasonics, Inc). The sample was centrifuged to separate the phases, and the acetone extract was saved. The extraction was repeated, and the extracts combined. Deionized water (5 mL) was added to the combined acetone extracts along with heptane (1 mL). The sample extract was shaken for 30 sec and centrifuged to separate the layers. The heptane extract was removed and reacted with 1 mL concentrated H_2SO_4. The heptane layer was removed, reacted with reduced copper powder to remove elemental sulfur, and then analyzed for PCBs.

Sediment extracts were analyzed for PCBs on a Hewlett-Packard 5890A gas chromatograph (GC) equipped with a splitless injection port, electron capture detector, and a 60-m fused silica column coated with a 0.25-μm coating of DB-5 (J and B Scientific, Inc.). The injector temperature was 270°C, and the detector temperature was maintained at 315°C. The column was held at 150°C for 1 min following injection, then programmed to 290°C at 1°C/min, and held at 290°C for 5 min. The output from the detector was collected on a Perkin Elmer LIMS 3210 computer.

Extracts were diluted prior to GC analysis with measured amounts of heptane containing octachloronaphthalene (OCN). The OCN served as an internal injection standard for peak identification.

The congeners comprising a peak were identified by injections of individual congener standards. For peaks for which standards were not available, identifications were based on retention times from the literature values.[7] The peaks utilized in this study, the corresponding congeners, and the method of identification are shown in Table 11.1.

Aroclor 1016 (A-1016) also was used by the capacitor manufacturing plant. Since A-1242 differs from A-1016 only by a slightly higher abundance of heavier PCB congeners, it is very difficult to differentiate between A-1016 and A-1242 when the higher-molecular-weight range of these mixtures is masked by A-1254. The problem of identifying A-1016 or A-1242 in the presence of A-1254 is even more difficult when dechlorination processes have altered distributions of congeners. To circumvent this problem we have assumed all releases of lower-molecular-weight PCB mixtures by the plant were A-1242. Our justification for this assumption is that A-1016 was substituted for A-1242 because of environmental concerns, and we presume Aroclor releases by the capacitor plant were greatly decreased or eliminated with the beginning of the usage of A-1016. Whether inputs were A-1242 or A-1016 makes little difference to the evaluations of the extent of dechlorinations seen in this study, but the time of the release of the mixture into the environment may affect estimates of dechlorination rates (see subsequent "Results and Discussion").

Quantitation of concentrations was done using external standards of A-1242 and A-1254. Spike and recovery tests of the procedure showed an average recovery of 106.3% (SD 23.7%) for individual peaks present in a mixture of Aroclor standards. Results were not corrected for recovery efficiencies. Triplicate analyses of a homogenate of a highly contaminated sediment using the described procedures showed that the coefficient of variation of the procedure was 7.6% in sediments that had a mean concentration of A-1242 plus A-1254 of 2960 ppm. Blanks were processed with sample sets and showed no contamination that interfered with the analysis of PCBs. All concentrations are on a dry weight basis. Concentrations as ppm (parts per million) refer to μg A-1242 plus A-1254/g sediment.

A computer program was developed to calculate the percentages of A-1242 and A-1254 content of samples from this study. This program used peak P039B as representative of A-1242 and peak P061 as representative of A-1254. These peaks were selected as representative of the Aroclor mixtures because they were found to be the most resistant to changes as a result of dechlorinations in this and another study.[5] Since these peaks are both present in A-1242 and A-1254, the computer program does a series of successive approximations and corrections to determine the percentages of these Aroclors in the residue.

To examine the effectiveness of this technique for estimating the percentages of A-1242 and A-1254 in sediment extracts, mixtures of A-1242 and A-1254 standards were analyzed and predictions of their relative quantities were made using the described technique. The results of these analyses are shown in the notation

$$(a{:}b - c{:}d)$$

Table 11.1. Peaks, Corresponding Congeners, and Structures

Peak ID	Tentative Identification of Congener	Structure
P005	10(S),4(S)	26–,2–2
P006	7(S),9(S)	24–,25–
CB006	6(S)	2–3
P008	5(S),*8(S)*	23–,2–4
CB019	19(L)	2–26
P013	12(S),13(S)	34–,3–4
CB018	18(S)	2–25
P015	15(S),17(L)	4–4,2–24
P016	24(L),27(L)	236–,3–26
P017	16(S),32(L)	2–23,4–26
CB026	26(S)	25–3
CB025	25(L)	24–3
CB031	31(S)	25–4
P024	*28(S)*,50(S)(M)	24–4,2–246
P025	20(S),21(S)(M),53(S),33(S)	23–3,234–,25–26,2–34
P025A	No I.D.	
P026	*22(L)*,51(L)	23–4,24–26
CB045	45(L)	2–236
CB039	39(L)(M)	35–4
P031	*52(S)*,73(L)(M)	25–25,26–35
CB049	49(S)	24–25
P037	44(S),104(S)(M)	23–25,26–246
P038	37(S),42(S),59(L)	3–34,23–24,3–236
CB072	72(S)(M)	25–35
P039A	No I.D.	
P039B	71(L)(M),*64(L)*,	26–34,4–236,2–234
CB040	40(S)	23–23
P044	100(S),67(L)	24–246,25–34
P045	58(L)(M),63(L)	23–35,4–235
P046	*74(L)*,94(L)(M)	4–245,26–235
P047	70(S),61(S)(M),76(L)	25–34,2345–,2–345
P048	66(S),93(S)(M),95(L)	24–34,2–2356,25–236
P049	*91(L)*,98(L)(M),55(L)(M)	24–236,23–246,3–234
P050	56(L)	23–34
P050A	60(S)	4–234
CB089	89(L)(M)(+ others 92,84)	26–234
P053	101(S),90(L)	25–245,24–235
CB099	99(L)	24–245
P055	150(L)(M),112(S)(M),*119(S)*	236–246,3–2356,34–246

Table 11.1, continued

Peak ID	Tentative Identification of Congener	Structure
P056	83(L),109(L)(M)	23–235,3–2346
P057	152(L)(M),*97(S)*,86(S)(M)	26–2356,23–245,2–2345
P058	*87(S)*,111(L)(M),115(S),81(S)(M)	25–234,35–235,4–2346,4–345
CB085	85(L)	24–234
CB136	136(S)	236–236
P061	77(S),*110(S)*	34–34,34–236
P064	151(S),82(L)	25–2356,23–234
P065	*135(L)*,124(L)(M),144(L)(M)	235–236,24–345,25–2346
P067	*107(L)*,108(L)(M),147(L)(M)	34–235,35–234,24–2356
P069	*149(L)*,106(L)(M),123(L)	245–236,3–2345,24–345
CB118	118(S)	34–245
P037	146(L),161(L)(M)	235–245–35–2346
CB153	153(S)	245–245
CB132	132(L)	234–236
CB105	105(S)	34–234
CB141	141(S)	25–2345
CB179	179(L)	236–2356
CB176	176(L)	236–2346
P082	138(S),163(L)(M)	234–245,34–2356
CB158	158(S)	34–2346
P088	187(S),182(S)(M),159(S)(M)	245–2356,246–2345,35–2345
CB183	183(S)	245–2346
P089	*128(S)*,167(L)	234–234,245–345
CB185	185(S)	25–23456
P093	*174(L)*,181(S)(M)	236–2345,24–23456
CB177	177(L)	234–2356
CB180	180(S)	245–2345
P106	170(S),190(L)	234–2345,34–23456

Table 11.1, continued

Notes: **Peak designation as P numbers are used for peaks containing coeluting congeners or where identifications are ambiguous. Identifications listed as tentative because standards for all congeners were not available and other congeners may coelute in the peaks listed. (S) identified with standard; (L) identified by comparison with literature values.[7] Congener (CB) numbering according to Ballschmiter and Zell.[22] Dominant congeners (believed to comprise ≥ 90% of peaks) are in italics. Congeners comprising <0.5% of Aroclors are identified by (M).[7] In structure column, numbers indicate position of chlorine atoms on each ring, and the dash represents separation of the two rings.**

where a = actual percentage A-1242 in standard mixture
b = actual percentage of A-1254 in standard mixture
c = predicted percentage of A-1242 from analysis of standard mixture
d = predicted percentage of A-1254 from analysis of standard mixture

The actual and predicted values showed good agreement (5.6:94.4-7.5:92.5), (27.5:72.5–26.2:73.8), (53.3:46.7–50.5:49.5), (77.4:22.6–74.7:25.3), and (95.6:4.4–93.7:6.3), which indicated the utility of this technique.

The data on the percentage mixture of A-1242 and A-1254 in sample extracts were used with relative concentration data for the peaks in A-1242 and A-1254 standards to reconstruct the original composition of the PCB mixtures that contaminated a specific sediment sample. The heights of peaks calculated to be in these original mixtures are called predicted values and are compared with measured values obtained from analyses of extracts from sediment samples. Comparative abundance plots are used to show the measured abundances (the abundance of congener X extracted from a sediment) relative to the predicted abundance (the abundance of congener X in the Aroclor mixtures which contaminated a specific sediment) and thereby show changes in PCB residues that have occurred since their release into the environment.

RESULTS AND DISCUSSION

The results of sediment analysis showed that PCB concentrations increased from south to north in upper NBH, and the highest concentrations were found nearest the electrical capacitor plant A (Figure 11.1, Table 11.2). In surface core sections (0–2.5 cm) in the upper NBH, PCB concentrations as totals of A-1242 plus A-1254 ranged from 102 ppm to 912 ppm. Surface sections of cores C6 and C7 from lower NBH had lower concentrations, 2.1 and 9.4 ppm. The sediment sample from Black Rock Harbor, CT, contained 21.4 ppm PCBs.

Concentrations in cores from the upper NBH generally increased with depth to the 15- to 17.5-cm section, then decreased in lower core sections. The highest concentration (2960 ppm) was found in the 15- to 17.5-cm section of core I11. Core C7 from lower NBH showed about the same PCB concentra-

Table 11.2. Concentrations and Percentages of Aroclor Mixtures in Sediments

Sample	Depth (cm)	µg A-1242 + A-1254/g(dry)	% A-1242	% A-1254
BRH	Unknown	21.4	42	58
I1101	0–2.9	912	69	32
I1123	5–7.5	2280	71	29
I1167	15–17.5	2960	56	44
I11XX	30–32.5	12	63	37
I11YY	45–47.5	3.2	68	32
I1401	0–2.5	740	72	28
I1423	5–7.5	1200	75	26
I1467	15–17.5	1720	76	24
I14XX	30–32.5	161	52	48
H1701	0–2.5	507	71	29
H1723	5–7.5	660	73	27
H1767	15–17.5	1560	75	25
H17XX	30–32.5	16.6	71	29
H2201	0–2.5	414	75	25
H2223	5–7.5	790	76	24
H2267	15–17.5	753	50	50
H22XX	30–32.5	5.7	56	44
H22YY	45–47.5	1	56	44
H2601	0–2.5	102	61	39
H2623	5–7.5	10	54	46
C601	0–2.5	2.1	39	61
C701	0–2.5	9.4	36	64
C723	5–7.5	7.4	37	63
C767	15–17.5	7.6	39	61
C7XX	30–32.5	7.6	51	49

Note: BRH = sediment from Black Rock Harbor, CT. Other names refer to locations in New Bedford—see map for locations.

tion (9.4–7.4 ppm) in sections from 0–2.5 to 30–32.5 cm. Core C6 and core H26 contained only trace amounts (< 0.2 ppm) of PCB in sections deeper than 0–2.5 cm (C6) and 5–7.5 cm (H26).

In some cores from the upper NBH, the percentage composition of A-1242 and A-1254 changed with depth. Cores I11 and H22 showed a relative increase in the percentage of A-1254 at the 15- to 17.5-cm section. Core I14 showed a similar change at the 30- to 32.5-cm section, but core H17 showed little change in the percentage Aroclor composition with depth. A small relative increase in the A-1254 composition was observed in the 5- to 7.5-cm section of core H26. The increases in percentage composition of A-1254 observed in lower core sections may reflect the history of inputs of Aroclor mixtures to the upper NBH. Although records of PCB purchases by the capacitor plant are incomplete prior to 1963, available records show A-1254 was used prior to 1963, A-1242 was used from 1963 to 1970, and A-1016 was used from 1970 to 1979.[4] The findings that Aroclor mixtures change differently from core to core and the presence of A-1242 and A-1254 at all depths may have resulted from differences in

1. depositional rates
2. percolation rates of PCB mixtures into the sediments
3. mixing of sediments by storms or biota

These results indicate that a historical record of PCB inputs to the upper NBH is not well preserved in some cores and underscores the difficulty in attempting to estimate rates of processes based on sedimentation rates at these locations. Core C7 shows a percentage composition lower in A-1242 and higher in A-1254 than found in the cores from upper NBH. The percentage composition of this core is about 38% A-1242 and 62% A-1254 for the top sections, but changes to 51% A-1242 and 49% A-1254 at the 30- to 32.5-cm section. This NBH core is located just inside the Hurricane Barrier and may have received inputs from other sources, such as capacitor plant B (Figure 11.1).

Substantial changes in the relative distributions of PCB congeners, which appear to be due to reductive dechlorinations, were found in many samples from upper NBH, but samples from lower NBH and BRH showed only small alterations. Comparisons of the relative distributions of PCBs were made using chromatograms of extracts, and using comparisons of the abundances of peaks or congeners in a sample with those present in the original mix of Aroclors that originally contaminated the sediment sample. Comparative abundance plots (CAPs) readily show which peaks are changing relative to the original inputs and are therefore useful to identify peaks that changed as a result of environmental processes. CAPs for a mixture of A-1242 and A-1254 (53:47) standards, core sections H2267 (15–17.5 cm), I1167 (15–17.5 cm), C767 (15–17.5 cm), and BRH sediment are shown (Figures 11.2–11.6). CAPs for the mixture of Aroclor standards showed only small changes between the predicted and measured abundances (Figure 11.2). For the H2267 core section, the bars to the left showed measured abundances below predicted values (less than zero) (Figure 11.3). These bars represent peaks containing congeners that are of relatively low molecular weight and are more volatile and more soluble than most of the other PCBs present. The decreased abundance of these congeners probably resulted from evaporation and/or dissolution of these congeners prior to incorporation of PCBs into consolidated sediment. At higher molecular weights, peaks CB072, P044, P055, CB179, and P088 show measured values that are two or more times greater than the predicted values. These peaks also increase in the sample from BRH and therefore may not be indicative of dechlorination processes. Notable decreases in the relative abundances of peaks P058, CB085, CB132, and CB105 are shown in the CAP for H2267, but similar decreases in the relative abundance of these peaks in the CAPs for BRH or for core section C767 (15–17.5 cm) from lower NBH were not observed. In the most highly dechlorinated sample, I1167 (15–17.5 cm section), the above peaks as well as other peaks (e.g., CB013, P045, P046,

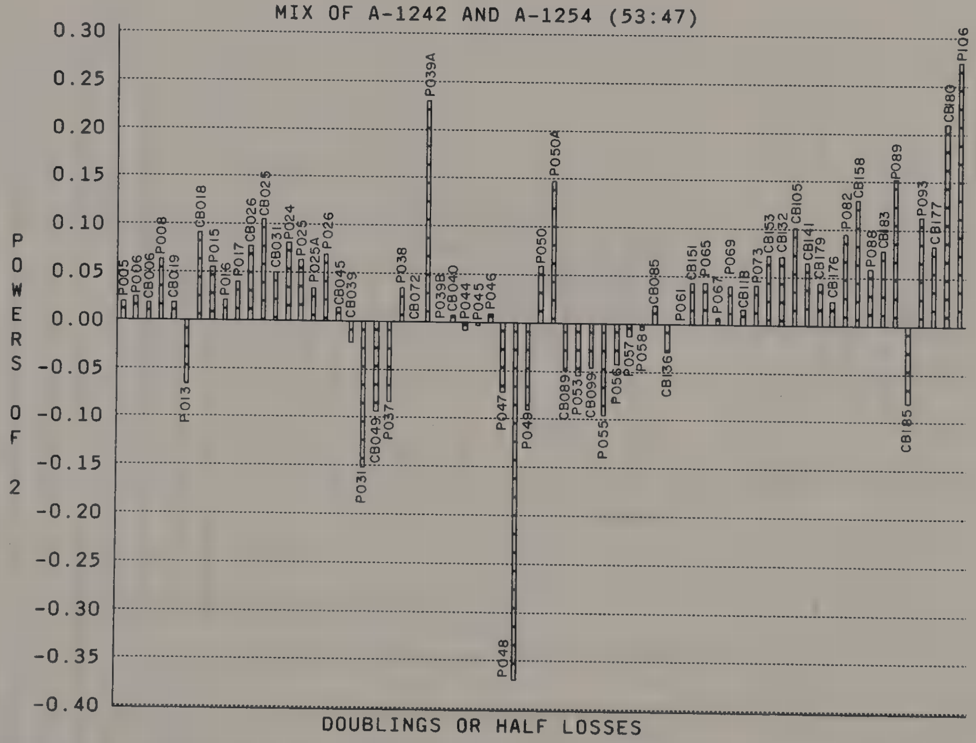

Figure 11.2. Comparative abundance plot for ■ 53:47 (weight:weight) mixture of A-1242 and A-1254 standards. Plot was made by determining the original mix of PCBs using peaks P039B and P061, which are representative of A-1242 and A-1254 inputs, respectively, but appear to be resistant to reductive dechlorinations. Measured abundances relative to the predicted starting mix of A-1242 and A-1254 are shown by the length of bars and is expressed to the power of two. Therefore, ■ value of 2 would indicate the measured abundance of that peak in the sediment was 4 times above the computer prediction of the abundance of that peak in the A-1242 and A-1254 inputs incorporated in the sediment. A value of -2 shows that the abundance of that peak is 0.25 of the computer prediction of the original input.

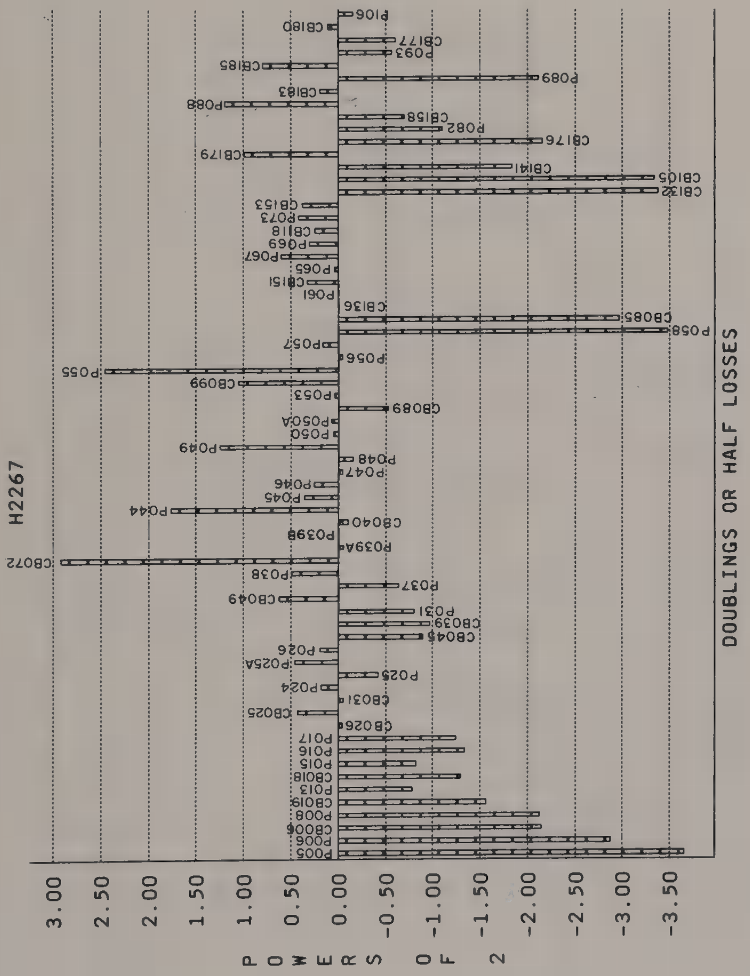

Figure 11.3. Comparative abundance plot for 15- to 17.5-cm section of core H22 (H2267). See Figure 11.2 for details.

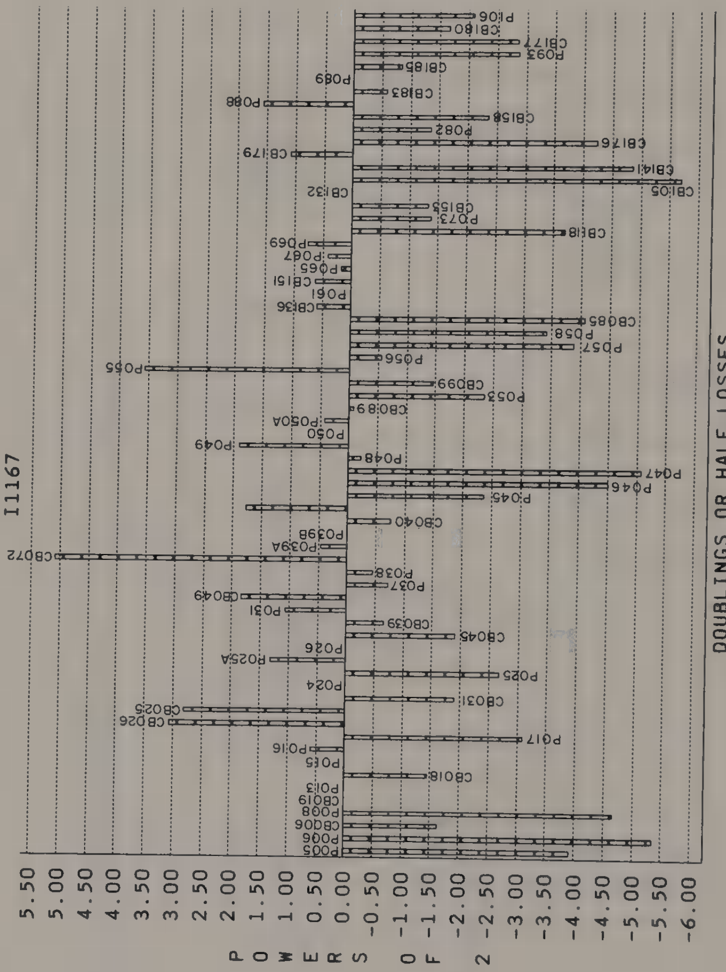

Figure 11.4. Comparative abundance plot for 15- to 17.5-cm section of core I11 (I1167). See Figure 11.2 for details.

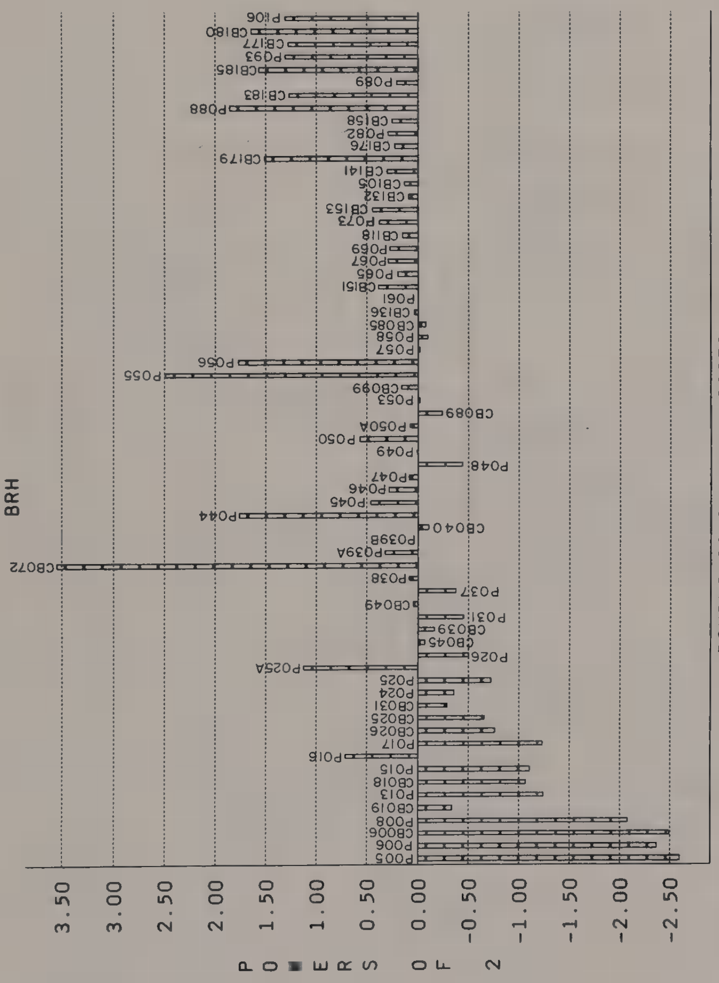

Figure 11.5. Comparative abundance plot for Black Rock Harbor sediment. See Figure 11.2 for details.

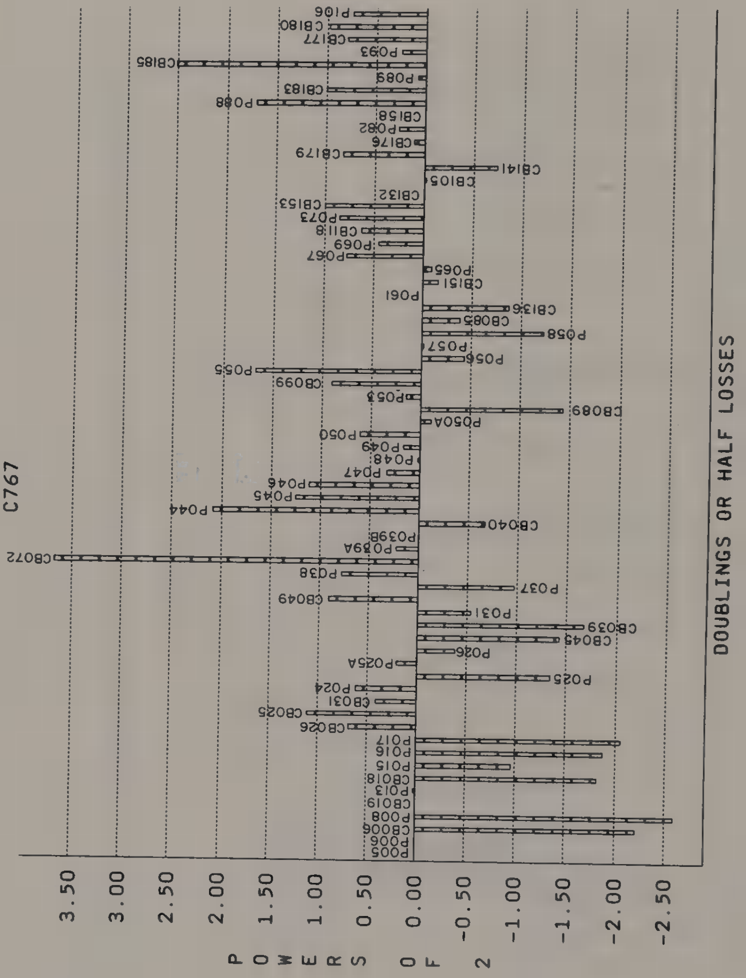

Figure 11.6. Comparative abundance plot for 15 to 17.5-cm section of Core C7 (C767). See Figure 11.2 for details.

P047, P053, CB099, CB153, and CB118) showed considerable decreases, while others (e.g., CB026, CB025) showed increases in relative abundance (Figure 11.4).

In general, the dechlorinations found in upper NBH sediments result in

1. decreases in the relative concentrations of selected higher-molecular-weight congeners, including some potentially toxic mono-*ortho* substituted congeners (e.g., CB105, CB118),[8] and other congeners known for their ability to bioaccumulate (e.g., CB99, CB101, CB153)[9]
2. increases in the relative concentrations of selected lower-molecular-weight congeners

Changes in PCB mixtures as a result of dechlorinations in the upper NBH resulted in PCB distributions that are lower in molecular weight, less bioaccumulatable, and less toxic as measured by capability to induce mixed-function oxidase enzyme systems.[9] These changes appear to be beneficial from an environmental perspective; however, large quantities of partially dechlorinated PCBs remain in upper NBH sediments, and the toxicities of the remaining mixtures are not known.

Plots of the relative abundance of peaks CB025 (structure 24–3), CB105 (structure 34–234), CB118 (structure 34–245), and CB153 (structure 245–245) across sediment samples demonstrate the variability in dechlorinations between locations, core sections, and congeners (Figures 11.7–11.10). These figures show the magnitude of increase or decrease in abundance of a peak measured in a sample relative to the predicted abundance of the peak in the mixture of A-1242 and A-1254 standards calculated as input for the sample.

The plot of the relative abundance of CB025 shows a decrease in the BRH sample, but increases of varying magnitude are observed in samples from upper and lower NBH. The congener comprising this peak (CB025) is a minor component of A-1242 and A-1254, and its increase in samples has been reported as indicative of a reductive dechlorination process.[3] Most of the samples from upper NBH show a factor of four or more increase in relative abundance of this congener. Sample I14XX (30- to 32.5-cm section) and all sections of core H22 show relative increases that are similar to those observed for lower NBH cores C6 and C7. Small increases in relative abundance of CB025 from the 0- to 2.5-cm and the 5- to 7.5-cm sections, to the 15- to 17.5-cm and 30- to 32.9-cm sections, are found in cores I11, and H17, but differences are not pronounced within cores except for the I14 30- to 32.5-cm section. The lower relative abundances of CB025 in I14XX and the H22 core may reflect conditions that are unfavorable to dechlorination or that retard dechlorination rates. The increase in relative abundance observed in C6 and C7 cores may demonstrate the initiation of dechlorination in these samples or may reflect down-bay transport and deposition of partially dechlorinated residues.

The plots of the relative abundance of peak CB105 shows only small changes for BRH, H2223 (5- to 7.5-cm section), and cores C6 and C7; how-

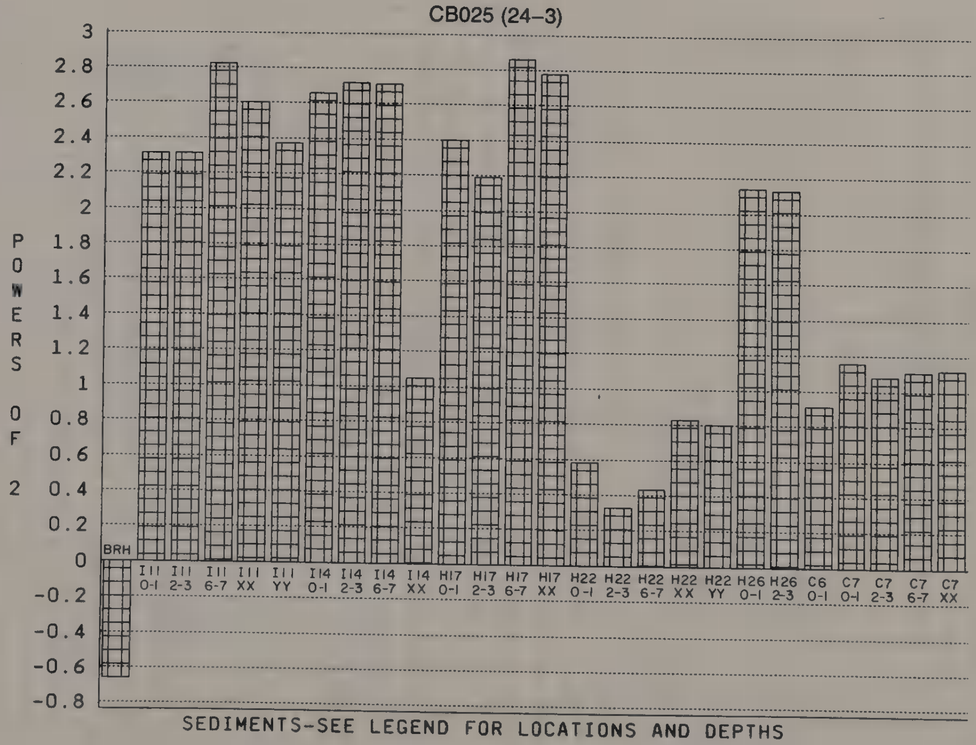

Figure 11.7. Magnitude of increase or decrease in abundance of peak CB025 (24–3) measured in a sample relative to the predicted abundance of that peak in the mixture of A-1242 and A-1254 standards calculated as input for the sample. Samples are identified as labels for bars, and numbers indicate depth of core sections in inches. Refer to Table 11.2 for section depths in centimeters. BRH sample is at extreme left. Cores from New Bedford Harbor are ordered (left to right) in increasing distance from the outfall of the capacitor plant (see Figure 11.1).

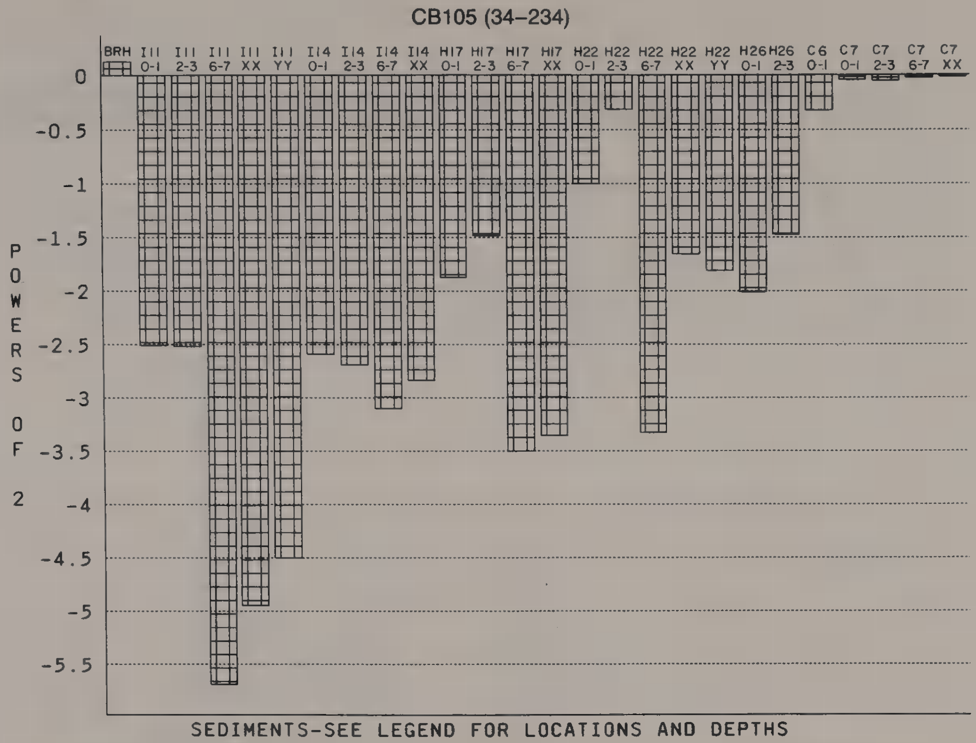

Figure 11.8. Magnitude of increase or decrease in abundance of peak CB105 (34–234) measured in a sample relative to the predicted abundance of that peak in the mixture of A-1242 and A-1254 standards calculated as input for the sample. See Figure 11.7 for details.

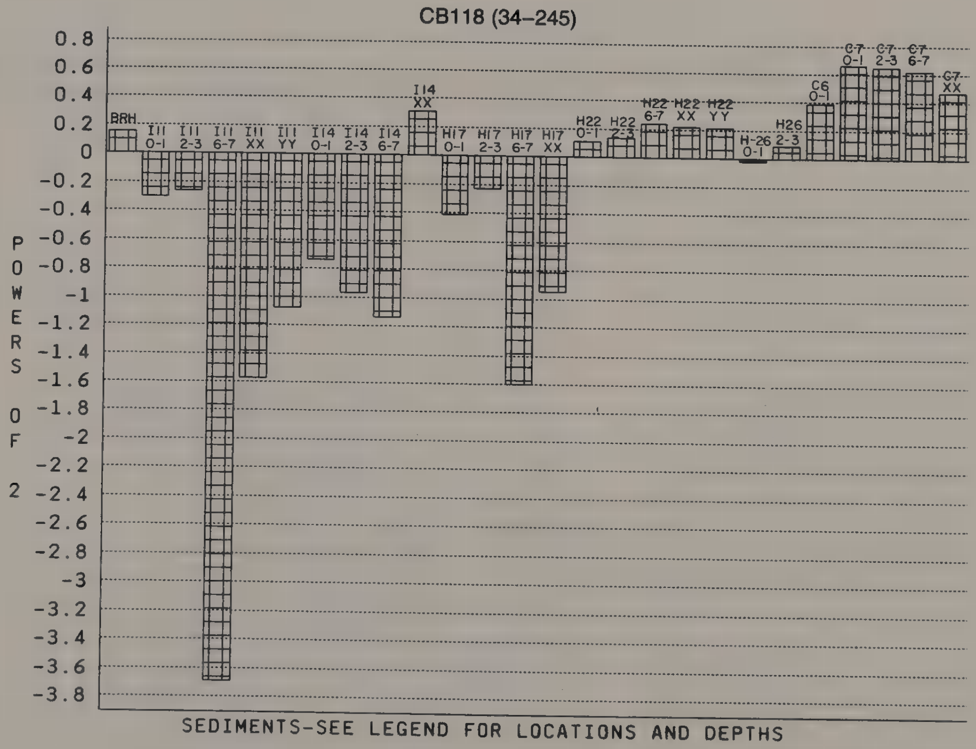

Figure 11.9. Magnitude of increase or decrease in abundance of peak CB118 (34–245) measured in a sample relative to the predicted abundance of that peak in the mixture of A-1242 and A-1254 standards calculated as input for the sample. See Figure 11.7 for details.

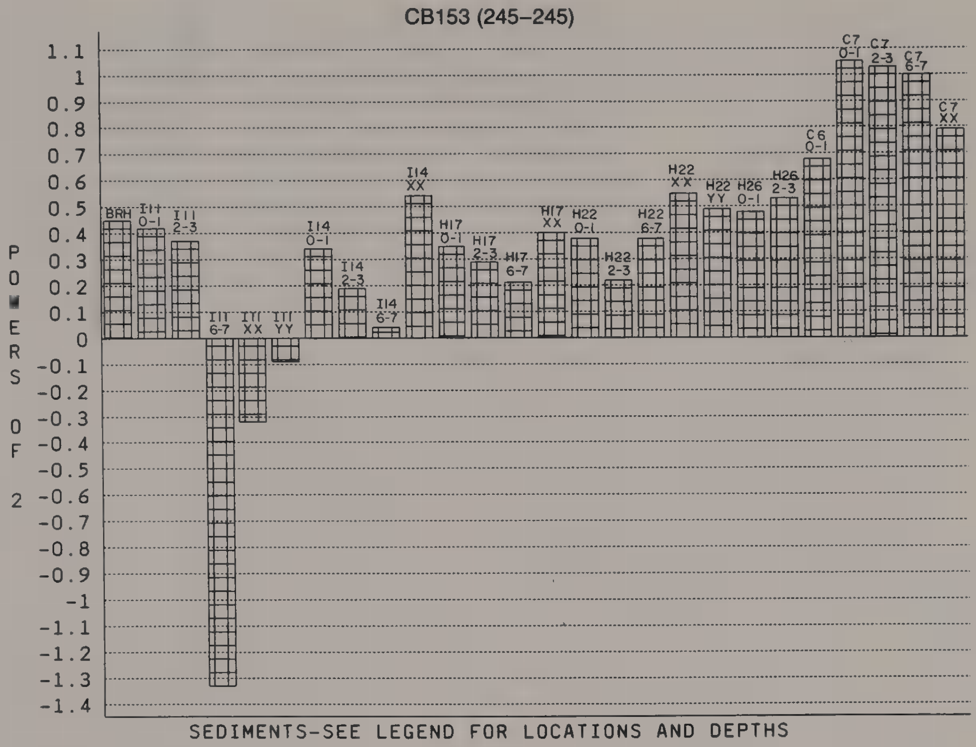

Figure 11.10. Magnitude of increase or decrease in abundance of peak CB153 (245–245) measured in a sample relative to the predicted abundance of that peak in the mixture of A-1242 and A-1254 standards calculated as input for the sample. See Figure 11.7 for details.

ever, samples from upper NBH show considerable decreases in abundance of this congener. Lowered relative abundance of CB105 also has been found to be indicative of dechlorination in sediments from the Hudson River.[1] For cores I11, H17, and H22, considerable decreases in relative abundance of CB105 are observed between the top (0–2.5 and 5–7.5 cm) and lower (15–17.5, 30–32.5, and 45–47.5 cm) core sections. These findings show residues are more dechlorinated near the plant and at depth in core sections than in core sections from lower NBH (cores C6 and C7).

Plots of the relative abundance of CB118 showed BRH, H22, H26, and lower NBH (C6 and C7) samples had small increases, but other upper NBH samples, except for I14XX (30- to 32.5-cm section), showed decreases. In upper NBH the greatest decreases were found in the 15- to 17.5-cm sections, and upper core sections (0–2.5 and 5.0–7.5 cm) showed smaller losses. Core section I1167 (15–17.5 cm) showed the greatest loss.

A relative abundance plot of CB153 shows an increase in this congener in all samples except for I1167, I11XX and I11YY (Figure 11.10). The greatest relative decrease was found in sample I1167.

Comparison of the relative abundance plots for these four compounds between the sediment samples shows that the dechlorination processes are not proceeding at equal rates or to equal extents. It appears from these data that the dechlorinations (which are presumably anaerobic microbial processes) may be a stepped series of dechlorinations, with each change in step occurring when the concentration of substrate falls below a suitable level. For example, all sections of core I11 show considerable decreases in relative abundance of CB105 (Figure 11.8), but the decreases for CB118 are much smaller in the 0- to 2.5-cm and 5- to 7.5-cm sections (Figure 11.9). For CB153, only core section I1167 (15–17.5 cm) shows large decreases in relative abundance (Figure 11.10). It appears that the dechlorinating organisms may have depleted the CB105 in sample I1167 (15- to 17.5-cm section), then switched to CB118, and then to CB153. The dechlorinations in other samples are less advanced in the stepwise process.

A more likely explanation for these distributions is that different bacteria are responsible for the dechlorinations. In this hypothesis, as a substrate (e.g., CB105) is completely utilized by one bacterial strain, another organism multiplies to utilize a different substrate (e.g., CB118). This hypothesis is supported by the results of other studies, which indicated that mixtures of bacterial populations or bacterial assemblages were responsible for dechlorinations in sediments from field sites.[10,11] In addition, studies conducted in the laboratory showed changes in growth conditions for bacteria resulted in different distributions of dechlorinated PCBs. The addition of cysteine hydrochloride was found to change the pattern of dechlorination.[12] The addition of biphenyl and maintenance in a N_2 atmosphere for 7 months enhanced degradation of highly chlorinated PCBs in laboratory cultures of PCB-contaminated sediments.[13] Different dechlorination products were found following addition of a trichlorobiphenyl to sediments and heating to 80°C for 30 min compared with maintenance at 24°C.[14] This difference indicates some bacteria may have been killed by the heating, which allowed another bacterial strain to take over the dechlorination process.

Another study of dechlorination in NBH found very little difference in the ratios of A-1242:A-1254 or extent of dechlorination between the upper (5.0–7.5 cm) and lower (15–17.5 cm) sections of individual cores taken in the intertidal zone.[5] They reasoned that the similarities of these core sections indicated the occurrence of an "active vertical transport (mixing) process within the upper estuary sediments." They suggest that this vertical movement may allow all the PCBs now in these sediments to reach the dechlorination zone and be dechlorinated. The present work sampled midchannel sediments in upper NBH and found differences in the proportions of Aroclors 1242 and 1254 and in the patterns and extents of dechlorination between the upper (5.0–7.5 cm) and lower (15–17.5 cm) sections of some cores (Figures 11.7–11.10). These results suggest that for midchannel sediments the vertical movement of PCBs may be more limited than in intertidal sediments. It appears that dechlorination of PCBs in these channel sediments may be limited by physical availability and/or by other factors needed for bacterial growth.

The plots for CB105, CB118, and CB153 (Figures 11.8–11.10) show the differences in the extent of dechlorination between congeners and sites and illuminate the difficulties in determining dechlorination rates. From these plots it is evident that to estimate rates of dechlorination, the congeners being dechlorinated and the sample location must be specified. A further difficulty in estimating rates of dechlorination in upper and lower NBH is that the history of PCB inputs to upper NBH have not been maintained in the sediments.

Although the Aroclor mixtures utilized (and presumably discharged) by the plant changed over the years of manufacture, distinct changes in inputs are not reflected in samples from sediment cores. PCBs were used as impregnation fluids in capacitors from 1947 until 1978. Plant records of PCB purchases are incomplete, but available records show A-1254 was utilized until 1963, when it was replaced with A-1242.[4] In 1971, A-1016 completely replaced A-1242 as an impregnation fluid.[15] Measurements of sediment depositional rates in NBH vary considerably depending on location and have increased substantially from a few millimeters/year to a few centimeters/year since construction of the Hurricane Barrier in 1966.[16] A computer model of upper NBH utilized to predict the distribution and fate of PCBs utilizes a sediment deposition rate of < 1 mm/year.[17] Another report estimates the sediment depositional rate at approximately 3 mm/year.[18] Using these estimates of depositional rates, the depth in the sediment corresponding to the change from A-1254 to A-1242 in 1963 would be ($1988 - 1963 = 25$ years) between 2.5 and 7.5 cm. As described earlier, depths of the changes from A-1254 to A-1242 varied in the core samples taken in upper NBH (depth to change was > 47.5 cm in core H17), but depths to the change were all in excess of the 2.5–7.5 cm estimated from depositional rates. It appears that the age of a contaminant in a sediment section within upper NBH many not be reliably estimated by using sediment depositional rates.

Although sediment deposition rates cannot be used to age PCB residues in cores from upper NBH, estimates of the average rates of dechlorination from input to time of sampling can be made by assuming the time of input of the Aroclor mixtures. Assuming an input of PCBs in 1963 and first-order kinetics, rate constants and half-lives were calculated for congeners CB031, CB105, CB118, and CB153 using sediment samples that showed smaller (H2267) and larger (I1167) changes as a result of dechlorinations (Table 11.3). For CB031, the rate constants are 0.001 (t^{-1}) and 0.053 (t^{-1}), and the half-lives are 465 years and 13.2 years for H2267 and I1167 samples, respectively. Considerable differences in half-lives were also observed between congeners within the same sample. For example, in sample H2267, CB031 has a half-life of 465 years, but CB105 has a half-life of 7.5 years. These differences emphasize that estimates of dechlorination rates vary greatly depending on the congener and the sample. The calculated rate constants represent averages over the 25-year time period from input to sample collection; other average rate constants and half-lives would be obtained if different input times were specified. The average rate constants do not give specific information regarding the past dechlorination rates. For example, the PCB congeners may have been dechlorinated over a short period of time, followed by years of dormancy. Further, these average rate constants offer no information on present dechlorination rates (if any) or if, and at what rate, these processes will continue in the future.

A variety of factors (e.g., availability of trace metals, nitrogen and minerals, carbon sources, PCB substrate, etc.) impact dechlorination rates in laboratory studies.[19,20] Another laboratory study found that dechlorination progresses under methanogenic, but not under sulfate-reducing conditions.[21] These factors, combined with the patchy distribution of PCBs in sediments of upper NBH, may be responsible for the large range of dechlorination rates estimated in this study.

CONCLUSIONS

1. PCBs in sediments from upper New Bedford Harbor showed considerable compositional alterations relative to predicted starting mixtures of Aroclors 1242 and 1254. These alterations included
    - a relative loss of lower-molecular-weight PCB congeners in all samples, presumably due to dissolution and evaporation prior to incorporation into sediment
    - relative decreases in the content of specific PCB congeners and the build-up of other congeners in some samples by processes that were presumed to be dechlorinations
2. The dechlorination processes varied in extent between samples, with the largest changes observed for samples closest to the outfall from the capacitor plant A in the 15- to 17.5-cm sediment core section. There was a trend toward less dechlo-

Table 11.3. Rate Constants and Half-Lives for Dechlorination of Selected PCB Congeners

	Rate Constants(t^{-1})		Half-Lives (years)	
Congener	H2267	I1167	H2267	I1167
CB031	0.001	0.053	465	13.2
CB105	0.092	0.16	7.5	4.4
CB118	[a]	0.10	[a]	6.8
CB153	[a]	0.04	[a]	18.8

[a] Peak showed increase in relative abundance; therefore, calculation of rate and half-life were not made.

rinated residues with distance from the capacitor plant A. Samples from lower NBH showed only small evidence of dechlorination, while reference samples from Black Rock Harbor, CT, showed none.

3. Samples with a lower extent of dechlorination showed relative decreases in abundance of specific congeners (e.g., P058, CB085, CB132, CB105). In more extensively dechlorinated samples, relative decreases in abundance of these and other congeners (e.g., CB031, P045, P046, P047, P048, P053, CB099, CB153, and CB118), and increases in abundance of congeners (e.g., CB026, CB025) that resulted from loss of chlorine atoms from more highly chlorinated congeners were observed. The potentially toxic mono-*ortho* congeners appear to be among those congeners most readily dechlorinated in upper NBH. Therefore, these dechlorination processes may have decreased the potential toxicity (as measured by mixed-function oxidase enzyme induction) of the PCB residues. However, large quantities of partially dechlorinated PCBs remain in the sediments of the upper NBH and the toxicities of these remaining mixtures are not known.
4. Considerable differences were observed in the calculated average rate constants for the dechlorinations depending on the sample and the congener.

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DISCLAIMER

Mention of product names does not constitute endorsement by the U.S. EPA.

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CHAPTER 12

Anaerobic Biotransformation of Halogenated Pesticides in Aquifer Slurries

Joseph M. Suflita, K. Ramanand, and Neal Adrian

INTRODUCTION

There are many physical, chemical, and biological factors that influence the fate of pesticides through soils and surface waters to groundwaters.[1] While these factors interact to mitigate the rate of pesticide migration, there is little doubt that such chemicals do contaminate subterranean aquifers. Not surprisingly, the entry of pesticides to groundwater reserves is associated with the manufacture, use, and disposal of these agricultural chemicals.[2]

Since much of the U.S. population relies on groundwater for drinking purposes, the fate of pesticides in aquifers is an area of intense ecological and toxicological concern. Persistence or dissipation of pesticides will help dictate the level of this concern. The persistence of compounds is, in turn, influenced by the metabolic abilities of the aquifer microbiota. The partial metabolism of pesticidal materials is sometimes of equal concern. Initial biotransformations can lead to the accumulation of intermediates with their own environmental and health impacts.

A variety of redox conditions can exist in aquifers, and the fate of pesticides should be evaluated in the context of the prevailing ecological conditions. Relative to aerobic biodegradation processes, little is known about the biotransformation of pesticides when oxygen is absent from aquifers. Under anaerobic conditions, pesticide metabolism could conceivably be linked to the consumption of other electron acceptors like nitrate, ferric iron, sulfate, or carbon dioxide.

This chapter summarizes several investigations on the predominant transformations of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2,4-dichlorophenoxyacetic acid (2,4-D), and bromacil and presents preliminary findings on the fate of propanil and linuron in anoxic aquifer sediments. We found that aryl reductive dehalogenation reactions represent an important fate process occurring during the breakdown of all of the pesticides examined in this study except linuron. Moreover, reductive dechlorination reactions were catalyzed under methanogenic but not under other redox conditions.

MATERIALS AND METHODS

The anaerobic fate of the pesticides 2,4,5-T, 2,4-D, bromacil, linuron, propanil, and related intermediates was evaluated in slurries obtained from an anoxic aquifer that was contaminated with leachate from a municipal landfill. Spatially distinct sulfate-reducing and methanogenic zones are known to exist within this aquifer.[3]

The samples were collected from the methanogenic zone, and the aquifer slurries were constructed in serum bottles as previously described.[4] Sterile filtered resazurin was added to the groundwater to a final concentration of 0.0002% and served as a redox indicator. Sterile sodium sulfide was added to a final concentration of 1 mM and ensured reducing conditions. The headspace of the bottles was adjusted to N_2/CO_2 (80%/20%) prior to the start of the experiment. Anoxic, filter-sterilized stock solutions of the pesticides were added to the slurries at initial concentrations of 125–500 μM. However, stock solutions of bromacil and linuron were made up in ether and added to sterile serum bottles before addition of sediment or groundwater. After evaporation of the ether, the serum bottles were transferred to an anaerobic glove box. Aquifer sediment and groundwater were then added as described previously.[4] The terminal electron acceptor status was experimentally manipulated by making appropriate amendments of either nitrate or sulfate (20 mM) to stimulate nitrate-reducing and sulfate-reducing conditions, respectively. Slurries that did not receive an exogenous amendment of an electron acceptor were designated the methanogenic incubations. Experiments were performed in at least duplicate and compared with autoclaved or substrate-unamended controls. All incubations were at room temperature in the dark. At intervals, samples were withdrawn by a syringe and stored at –10°C until analysis.

The disappearance of the parent substrates and the accumulation of the intermediate products were monitored on the liquid phase of the aquifer slurries by modifications of previously published HPLC methods.[4,5] The isocratic mobile phases for the various separations consisted of different ratios of 50 mM sodium acetate buffer (pH 4.5) and acetonitrile at a flow rate of 1.2–1.5 mL/min. All compounds were detected by UV absorbance with a variable wavelength detector (Beckman model 165, Beckman Instruments, Inc., Berkeley, CA) operated at 280 or 254 nm. Identification and quantification of the compounds were performed by comparing retention time and integrator responses, respectively, with external standards.

The methane production was detected by gas chromatography.[3] An evaluation of the fate of pesticides, intermediates, and end products was made relative to both autoclaved and substrate-unamended controls. The identity of individual metabolites was minimally based on their chromatographic behavior. However, the identity of the most important intermediates was confirmed by ether extraction and subsequent analysis by GC-MS using a Hewlett-Packard GC 5890 equipped with DB-5 fused silica capillary column and a model 5970 mass selective detector[6] or by LC-MS using a Kratos MS25RF mass spectrometer equipped with a Vestec liquid chromatography ionization interface.[5]

RESULTS AND DISCUSSION

2,4,5-T and 2,4-D

The herbicides 2,4-D and 2,4,5-T (2,4-dichloro- and 2,4,5-trichlorophenoxyacetic acid, respectively) are often used in combination with each other and are ingredients of many pesticide formulations. These chemicals have a relatively high water solubility, are only slightly retained in soil, can contaminate aquifers, and the trichlorinated derivative tends to resist aerobic microbial destruction.[7]

2,4,5-T and 2,4-D were reductively dehalogenated to lesser halogenated congeners in methanogenic aquifer slurries (Figure 12.1); that is, the *para* or *meta* halide of 2,4,5-T or either chlorine of 2,4-D was replaced by a hydrogen atom. The first detectable metabolite observed during 2,4-D metabolism was 2,4-dichlorophenol, while 2,5-or 2,4-dichlorophenoxyacetate were formed from 2,4,5-T. The latter compounds could then be converted to monochlorophenoxyacetate derivatives or to dichlorophenols and eventually to monochlorophenols and to phenol under methanogenic conditions (Figure 12.1).[4,6] No parent substrate transformation was noted in autoclaved controls, and no comparable intermediates could be detected in substrate-unamended controls.

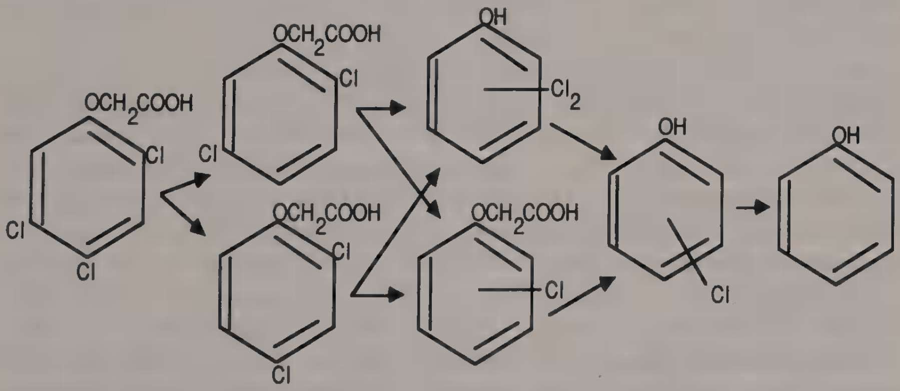

Figure 12.1. Proposed pathway for the anaerobic decomposition of 2,4,5-T and 2,4-D in methanogenic aquifer slurries. Adapted from Gibson and Suflita.[4]

The transient accumulation of the three monochlorophenol isomers but not of phenoxyacetate suggested that the complete removal of all chlorines could not proceed in the presence of an ether-bonded substituent. The ether cleavage always preceded the last halogen removal from the aromatic ring. The pathways for 2,4-D and 2,4,5-T converged on phenol. In the absence of oxygen, the latter compound can be mineralized under a variety of redox conditions.[8-10]

No significant alteration of the parent herbicides was noted in parallel experiments using aquifer slurries sampled from the sulfate-reducing portion of the same aquifer.[4] Several di- and monohalogenated phenolic compounds, which were potential intermediates of the herbicides, acted similarly when they were used as parent substrates. However, such halophenols could only be reductively dehalogenated in methanogenic incubations.[4] The exogenous addition of sulfate to methanogenic aquifer slurries drastically inhibited the dehalogenation of 2,4,5-T.[6]

Bromacil

Bromacil is a moderately to highly mobile herbicide that has been known to persist for up to 2 years after application.[11] It has been shown to leach through soils[11] and is a known contaminant of groundwater supplies.[12] Bromacil is a halogenated nitrogen heterocyclic compound. Heterocyclic compounds are in widespread use and are frequently detected as aquifer contaminants.[13] However, the anaerobic metabolic fate of such materials is poorly understood.[14]

In our studies, liquid chromatographic analysis of methanogenic aquifer slurries amended with bromacil revealed that the parent substrate disappeared from nonsterile samples (Figure 12.2). The loss of the parent substrate was coincident with the appearance of a more polar compound with a shorter HPLC retention time (Figure 12.2). The latter compound was not detected in slurries at the start of the incubation or in substrate-unamended controls. However, trace amounts of a compound which cochromatographed with the bromacil intermediate were detected in autoclaved controls at the end of a 5-month incubation period.[5] Presumably, this reflects the small amount of bromacil that can be abiotically transformed in these incubation systems. No significant degradation of the parent herbicide occurred when the aquifer slurries were incubated under nitrate-reducing or sulfate-reducing conditions.

Based on suggestions in the literature and by analogy to the proposed fate of other nitrogen heterocyclic herbicides, we suspected that bromacil might be hydrolytically dehalogenated.[7,15] Therefore, the presumed metabolite was isolated by HPLC, rechromatographed to verify purity, and subjected to LC-MS analysis.[5] The metabolite resulting from hydrolytic dehalogenation of bromacil should display molecular ion peaks at 199 and 216. However, the thermospray mass spectrum of the metabolite exhibited molecular ion peaks at m/z 183 and 200. Further, unlike the parent substrate, the metabolite molecular ion peak did not exhibit a bromine isotopic abundance pattern. This LC-

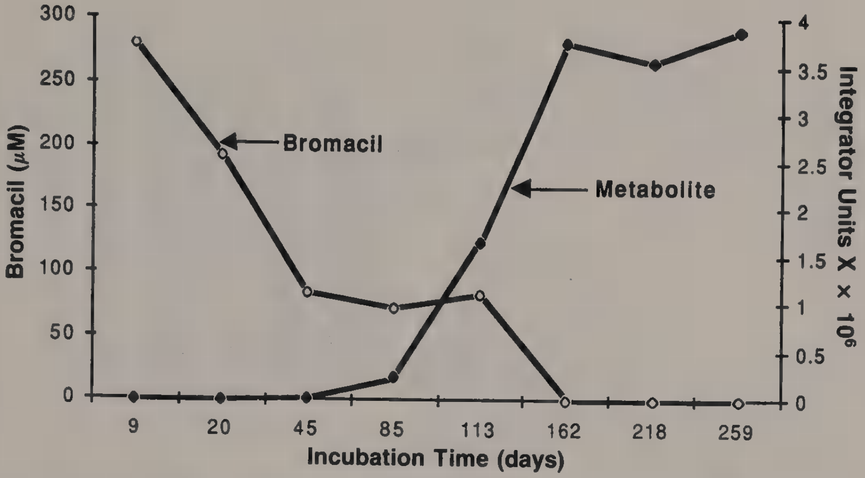

Figure 12.2. The degradation of bromacil and the production of a metabolite in methanogenic aquifer slurries. Adapted from Adrian and Suflita.[5]

MS analysis allowed us to identify the metabolite as 3-sec-butyl-6-methyluracil (Figure 12.3). Thus, like the homocyclic halogenated herbicides considered above, bromacil was reductively debrominated, but only under methanogenic conditions. The metabolite was not transformed with further incubation, but studies to clarify the fate of this chemical under other redox conditions have been initiated.

Propanil

Propanil (3,4-dichloropropionanilide) is predominantly used with rice (*Oryza sativa*) and other agricultural crops to control weeds.[16] The metabolic fate of the dihalogenated herbicide has been extensively studied under aerobic conditions.[17,18] Information on the fate of propanil under anaerobic conditions is limited but suggests that propanil may undergo either hydrolysis[16] or reductive dechlorination[19] (Figure 12.4). Propanil can conceivably contaminate groundwater reserves because of its high water solubility. Studies on the anaer-

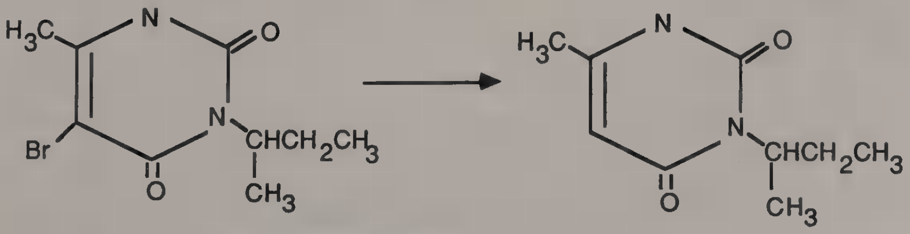

Figure 12.3. The reductive dehalogenation of bromacil to 3-sec-butyl-6-methyl-uracil under methanogenic conditions.

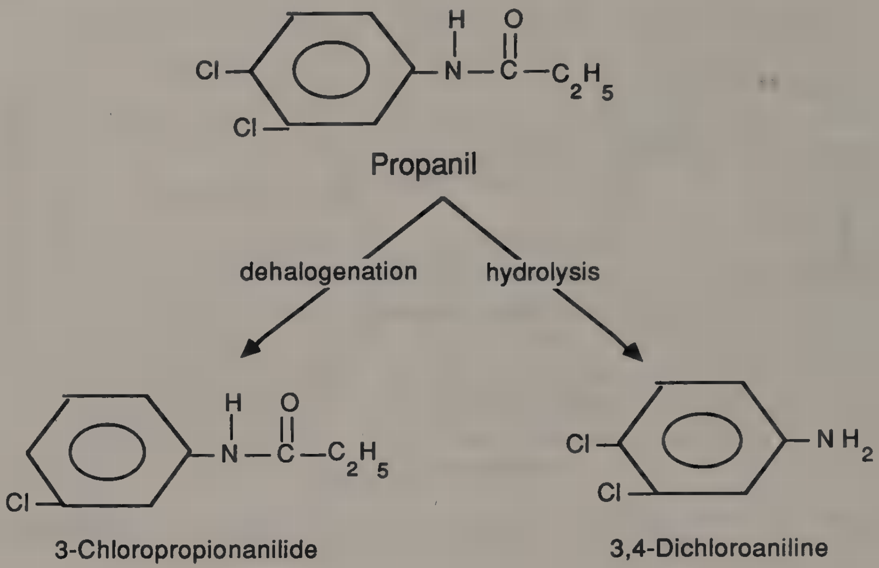

Figure 12.4. The presumed metabolic fate of propanil in anoxic environments.

obic fate of propanil is restricted to flooded soils and sediments, and no information is available about its behavior in anoxic aquifers. We evaluated the biodegradation of propanil in anoxic aquifer slurries.

The fate of the herbicide was studied in methanogenic, sulfate-reducing, and nitrate-reducing incubations. Propanil was biologically transformed under all three redox conditions in active samples (Figure 12.5); no significant loss of the compound was observed in autoclaved controls (data not shown).

The decomposition of propanil was greater in nitrate-reducing incubations than in other redox conditions. After a lag of about 6 days, 124 μM levels of the parent material were reduced to nondetectable levels by 34 days under nitrate-reducing conditions. During the same period, about 30–35% of the parent compound was still present in corresponding methanogenic or sulfate-reducing incubations (Figure 12.5).

Concomitant with the loss of propanil was the accumulation of another compound in all three types of incubations. This intermediate was more polar and had a shorter retention time than the parent compound when analyzed by HPLC. Chromatographic evidence was used to tentatively identify this compound as 3,4-dichloroaniline. Thus, propanil was hydrolytically cleaved as the primary anaerobic biotransformation reaction to form 3,4-dichloroaniline under all three incubation conditions (Figure 12.5). Similar results were obtained with anaerobic enrichment cultures obtained from a nonflooded soil, but the ecological conditions for the hydrolysis reaction were not delineated.[16] The haloaniline accumulated to near stoichiometric amounts during the early stages of the incubation but did not substantially change with additional time.

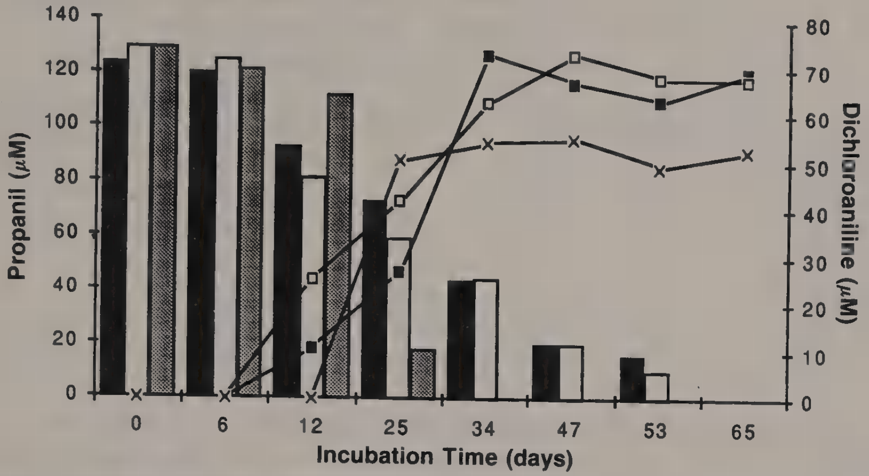

Figure 12.5. The disappearance of propanil *(bars)* and the appearance of 3,4-dichloroaniline *(points)* under methanogenic *(solid black)*, sulfate-reducing *(solid white)*, and nitrate-reducing *(shaded, X)* conditions.

Since propanil continued to disappear after 34 days, it may be that another propanil transformation product was formed but remained undetected in our HPLC analysis. Further work is being directed to clarify this point.

The 3,4-dichloroaniline intermediate was not detected in active aquifer slurries at the start of the incubation or in substrate-unamended controls throughout the period of study. However, after 5 months of incubation, we observed the formation of small amounts of this haloaniline (< 5%) in autoclaved samples, presumably as a result of the abiotic hydrolysis of propanil. The 3,4-dichloroaniline was not significantly altered in either sterile or nonsterile aquifer slurries with continued incubation.

The fate of 3,4-dichloroaniline in sediment slurries obtained from the same anoxic aquifer was examined in previous experiments.[20] Under methanogenic conditions, 3,4-dichloroaniline as well as tri- and tetrachloroanilines were reductively dehalogenated over a 8-month incubation. The same compounds were not transformed in sulfate-reducing incubations. The *para* chlorine was reductively removed from the 3,4-dichloroaniline to result in the accumulation of 3-chloroaniline.[20] The latter compound as well as its isomeric counterparts remained persistent under the test incubation conditions when they were used as parent substrates.[20]

3,4-Dichloroaniline and 3-chloroaniline may result from the anaerobic destruction of propanil as a result of initial amide hydrolysis of the parent herbicide followed (in the case of the monohaloaniline) by a reductive dehalogenation step. Consistent with the above suggestion is the finding of both 3,4-dichloro- and 3-chloroaniline as products of propanil decomposition in rice paddy soil.[16]

Table 12.1. The Disappearance of Linuron from Both Sterile Control and Experimental Aquifer Slurries Incubated under Different Redox Conditions

Incubation Time (days)	Linuron Remaining Under Various Incubation Conditions (% of initial substrate concentration)					
	Methanogenic		Sulfate-Reducing		Nitrate-Reducing	
	Exptl	Control	Exptl	Control	Exptl	Control
19	100	100	100	100	100	100
37	72	—	43	—	96	—
46	59	—	9	—	79	—
56	21	106	1	69	84	58
121	0	98	1	—	85	49
209	0	91	0	86		
306	0	60	0	55		

The addition of sulfate inhibited the dehalogenation of haloanilines in a manner that was comparable to the effect of this anion on the chlorophenols and chlorophenoxyacetates.[20,21] It is not yet known whether the reductive dehalogenation of chloroanilines is possible under nitrate-reducing conditions. The nitrate-dependent biotransformation of 3,4-dichloroaniline and monochloroanilines has been previously shown,[22,23] but the metabolic pathways involved were not elucidated.

Linuron

Substituted urea herbicides including linuron are used extensively for weed control with a wide variety of horticultural and agricultural crops.[24] Sediments of lakes, ponds, irrigation ditches, and other aqueous impoundments are often repositories for many such chemicals added to the environment.[19] There are conflicting reports in the literature concerning the fate of linuron in the environment,[19,25–27] although it is thought that the loss of this compound is predominately through microbiological processes.[27] Since it was previously reported that linuron could be reductively dehalogenated,[19] we undertook a study to clarify the fate of this 3,4-dihalogenated phenylurea herbicide in anoxic aquifer sediments.

We studied the degradation of linuron in aquifer slurries under methanogenic, sulfate-reducing, and nitrate-reducing conditions. Linuron was rapidly degraded under methanogenic and sulfate-reducing conditions, but only slowly transformed under nitrate-reducing conditions. After 56 days of incubation, 80 and 99% of the applied linuron (about 120–150 μM) had disappeared in the methanogenic and sulfate-reducing incubations, respectively (Table 12.1). During the same time frame, only about 15% of the linuron had disappeared in the bottles incubated under nitrate-reducing conditions. After 121-days incubation there was no detectable linuron in the bottles incubated under methanogenic conditions, and only 1% remained in the bottles incubated under sulfate-reducing conditions. However, 85% of the linuron remained in the nitrate-reducing incubations (Table 12.1).

Concomitant with the disappearance of linuron was the appearance of another compound that exhibited a shorter HPLC retention time. A comparison of HPLC and GC retention times of this compound with previously published transformation products tentatively identified the compound as 3-(3,4-dichlorophenyl)-1-methylurea. The compound was derivitized (ethylated) and analyzed by GC-MS.[28] The mass spectrum of the derivitized metabolite contained two predominant peaks at m/z 58 and 86. The mass spectrum of the derivitized transformation product was identical to that of authentic 3-(3,4-dichlorophenyl)-1-methylurea that was similarly derivitized and analyzed. In addition, these two spectra were consistent with the previously published spectral profile of this compound.[28] Therefore, linuron was demethoxylated in the anoxic aquifer sediments (Figure 12.6). After 200 days there was no detectable linuron in the experimental bottles incubated under methanogenic or sulfate-reducing conditions. During this time, there was about 10 and 15% loss of linuron from the methanogenic and sulfate-reducing sterile controls, respectively.

Nevertheless, desmethoxylinuron was easily detected in sterile controls. This transformation product steadily increased with longer incubation times. The formation of this compound may be the result of the slow abiotic destruction of linuron. Stepp et al. have also observed the formation of demethoxylated linuron in sterile controls.[19]

One must cautiously interpret such data since autoclaved controls are almost certainly not as reducing as experimental incubations. It may be that this particular type of transformation is favored under the highly reducing conditions routinely encountered in methanogenic or sulfate-reducing incubation. In our experiments, the desmethoxylinuron reached a maximum when linuron could no longer be detected in experimental incubations. The area of the peak did not substantially change with further incubation, suggesting that

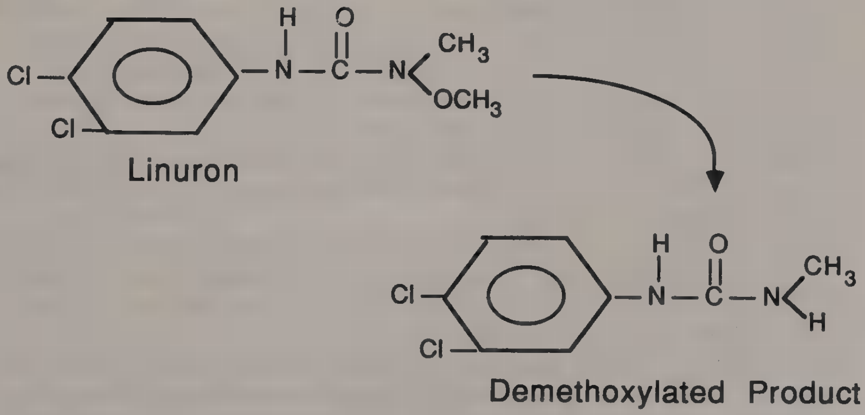

Figure 12.6. The proposed conversion of linuron to desmethoxylinuron in both sterile and nonsterile incubations under anaerobic conditions.

demethoxylated linuron tends to resist further anaerobic destruction. Experiments are planned to address this possibility.

CONCLUSIONS

Under anaerobic conditions, reductive dehalogenation seems to be a predominant process for the removal of halogens from homocyclic or heterocyclic pesticides. Examples of environmental chemicals that are susceptible to such transformations include 2,4-D, 2,4,5-T, and bromacil, as well as a variety of halogenated phenols and anilines that were formed as intermediates during the metabolism of the parent molecules. Methanogenic conditions seem best suited for reductive dehalogenation reactions, but such bioconversions may be possible under other anaerobic conditions. Propanil was hydrolytically cleaved as the primary degradative event under all the test anaerobic incubation conditions, whereas linuron was demethoxylated in both sterile and nonsterile aquifer slurries. This work helps to clarify the fate of polluting organic chemicals that enter or reside in anoxic aquifers.

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CHAPTER 13

Reductive Dechlorination of Dichlorophenols in Anaerobic Pond Sediments

Dorothy D. Hale, John E. Rogers, and Juergen Wiegel

INTRODUCTION

A number of aliphatic and aromatic compounds, including xenobiotics, are biodegraded anaerobically.[1-10] Within the past decade, significant progress has been made in understanding the anaerobic biodegradation of organics. Initially, pure or mixed anaerobic cultures of photosynthetic, respiratory, and fermentative microorganisms were investigated.[9,10] More recently the biodegradation of a variety of organic compounds has been studied in samples from several anaerobic sediments, soils, aquifer materials, and sewage sludges under methanogenic conditions.[7-9,11-14]

Chlorinated phenols, which are listed as priority pollutants by the U.S. Environmental Protection Agency,[15] are biodegraded in a number of anaerobic ecosystems.[8,11-14] The initial transformation in the anaerobic biodegradation of chlorophenols is, with few exceptions, reductive dechlorination to phenol. Phenol may then be degraded either through cyclohexanol, cyclohexanone, and adipate to succinate, propionate, and acetate[9,10] or through benzoate and acetate to the end products, carbon dioxide and methane.[16-18]

Much of the early work in our laboratory has concentrated on the degradation pathways of chlorinated phenols. More recently we have directed our studies toward understanding the environmental factors and chemical characteristics that influence the fate of these compounds in anaerobic ecosystems. In this chapter, we present results of experiments designed to examine the effects of environmental parameters on the reductive dechlorination of chlorinated phenols. Research in our laboratory has focused on this reaction because reductive dechlorination is usually the initial step, and possibly the rate-limiting step,[18] in the anaerobic degradation of these compounds.

Table 13.1. Persistence of Dichlorophenols in Anaerobic Sediment Slurries from Five Ponds

	T_{50} (days)				
Dichlorophenol	**Cherokee**	**Bolton's**	**Bar H**	**Sandy Creek**	**2-Boat**
2,3–	20 (17)	34 (98)	36	39	29
2,4–	21 (20)	21 (56)	21	21	21
2,5–	33	47	>98	61	>98
2,6–	34 (21)	>98 (224)	>98	>98	>98
3,4–	>84	>84	>84	49	>84
3,5–	35	42	>84	48	21

Note: Numbers represent the time for dechlorination of 50% of a dichlorophenol to a monochlorophenol. Numbers in parentheses represent the T_{50} for a dichlorophenol after its intial addition to sediments from Cherokee and Bolton's Ponds in a second study of microbial dechlorinating activity.[13]

BACKGROUND

To determine the prevalence and specificity of microbial chlorophenol dechlorinating activity in anoxic freshwater sediments, we initially studied the reductive dechlorination of the six dichlorophenols (DCPs) (2,3-, 2,4-, 2,5-, 2,6-, 3,4-, and 3,5-DCP) in sediments from five ponds in the Athens, Georgia, area. Although dechlorination of several isomers was evident in sediments from each of the ponds, a range of from 20 to more than 98 days was required for transformation of 50% of the DCPs to the monochlorophenols (T_{50}, Table 13.1). Sediment microorganisms in Cherokee Pond exhibited the highest dechlorinating activity, as evidenced by T_{50} values for the DCPs of from 20 to 35 days. The 3,4-DCP was an exception with a T_{50} of $>$ 84 days. In contrast, dechlorinating microorganisms in the other four pond sediments were less active toward the DCPs, as reflected in the longer T_{50} values for each of the isomers. In the five sediments, chlorines were removed from the DCPs in the order *ortho* $>$ *meta* $>$ *para*. A similar order of dechlorination was determined for the monochlorophenol products.

The DCP dechlorinating activity present in the most active sediment (Cherokee Pond) and in a less active sediment (Bolton's Pond) were investigated further to examine the extent of the difference in DCP dechlorinating activity of microorganisms in these pond sediments.[13] The dechlorinating activity of the anaerobic sediment microorganisms was examined in sediment slurries repeatedly exposed to 2,3-, 2,4-, or 2,6-DCP. After the final addition of DCP, subsamples of these slurries were individually exposed to each of the six DCP isomers to determine substrate specificity.

Following the initial addition of DCP, a period of adaptation was observed in sediments from both ponds. No significant loss of DCP or production of monochlorophenol occurred during this time. The adaptation periods, ranging from 12 to 14 days in Cherokee sediments and from 35 to 196 days in Bolton's sediments, were similar to those observed in the initial study of five pond sediments. Likewise, T_{50} values for the DCPs in Cherokee sediments were similar to those determined initially (Table 13.1). However, T_{50} values for the three isomers differed two- to threefold from the initial T_{50} values in Bolton's sediments and three- to tenfold between sediments from the two ponds. Adaptation periods were not detectable following a second addition of DCP. Repeated addition of 2,3-, 2,4-, or 2,6-DCP to sediments adapted the indigenous microorganisms to initially remove the *ortho* chlorine without a lag. Adapted Cherokee sediment microbes exhibited faster dechlorination rates and a broader substrate specificity than adapted Bolton's sediment microorganisms (data not shown).

The disparity in DCP dechlorinating activity and substrate specificity of the microorganisms in Cherokee and Bolton's pond sediments exemplifies the difficulty faced in predicting the persistence and fate of some hazardous compounds in similar anoxic environments. The task is further complicated by temporal and spatial variation in sediment characteristics within, as well as between, sites in such environments. In order to address this problem, a study was initiated to monitor selected sediment properties and microbial dechlorinating activities of sediments collected every other month for a year from five sites in Cherokee Pond (Figure 13.1). Experiments were designed to determine correlations between selected sediment properties and rates of dechlorination of 2,4-, 2,5-, and 3,4-DCP. Our objective was to utilize the correlations to predict the persistence in anoxic sediments of chlorophenols with various chlorine substitution patterns.

MATERIALS AND METHODS

Sediment (0–10 cm) and overlying water were collected every other month from May 1988 to May 1989 from selected locations in five sites in Cherokee Pond. The samples were collected in sterile wide mouthed quart Mason jars and Erlenmeyer flasks, respectively, after determination of in situ temperature. The samples were transported to the laboratory and placed into an anaerobic chamber, with an atmosphere 95% N_2:5% H_2. All subsequent manipulations of the sediment and water samples were performed in the chamber. Sediments were sieved and stored as previously described.[13]

Serum bottle microcosm studies were conducted as follows. Slurries of 20 mL (10% dry sediment w/v) were prepared with sediments and site water in 100-mL serum bottles, which were capped with butyl rubber stoppers and crimp sealed. Experiments were initiated by addition of 1 mL of an aqueous stock solution (200 mg/L) of a DCP to the reaction vessels to yield a final concentration of 10 mg/L. Incubation and sampling of serum bottles and high pressure liquid chromatography of slurry extracts have been described previously.[13]

Sediment pH and redox potential (E_h) were determined as follows. Direct pH determinations were made with an Orion Research digital pH/millivolt meter (Model 611) with a combination pH electrode (Orion 910500). The

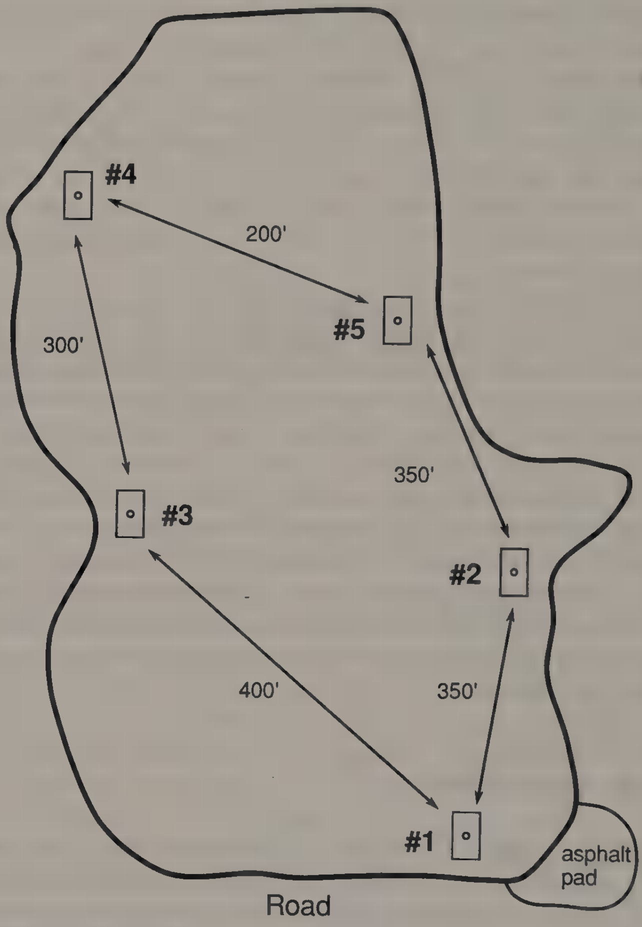

Figure 13.1. Location of the five sediment sampling areas (10 ft x 30 ft) in Cherokee Pond.

electrode was calibrated with pH 4 (potassium biphthalate) and pH 7 (potassium phosphate monobasic-sodium hydroxide) buffers (Fisher Scientific). Redox potential measurements were made for each sediment using a platinum/Ag/AgCl combination electrode (Orion 977800). The redox reference solution was a 0.1 M ferrous ammonium sulfate/0.1 ferric ammonium sulfate in 1.0 M sulfuric acid solution.[19]

Sulfate and nitrate concentrations in water samples were determined with a Dionex 2020i ion chromatograph utilizing an HPIC AS-4 analytical column and electrometer. The standard eluent, run at 1.5 mL/min, was 0.003 M sodium bicarbonate/0.0024 M sodium carbonate. A 0.025 N sulfuric acid solution was used with the continuous flow fiber suppressor. Water samples were injected through a 0.22-μm filter onto a 100-μm loop. A Hewlett-Packard

3390A integrator was used to quantify sulfate and nitrate by comparison to sodium sulfate and potassium nitrate standards, respectively.

Organic carbon analyses were performed on sediment samples ground to a powder with a mortar and pestle after being dried at 105°C and on water samples filtered through glass microfiber filters (Whatman GF/F, 0.7 μm). Persulfate oxidation of the samples followed the protocol for determination of carbon in sediments, soil, and water, as described in the procedures manual of the Oceanography International Corporation, Model 524C carbon analyzer. The carbon dioxide evolved from the samples was quantified by comparison to potassium biphthalate standards using a Horiba PIR-2000 infrared gas analyzer and integrator.

Numbers of 2,4-DCP dechlorinating microorganisms in Cherokee sediments were estimated with a compound-specific most probable number (MPN) assay. Dilutions of 10^{-1} to 10^{-5} were prepared first by serial dilution of 0.5 mL of slurry into 4.5 mL of site water which had been filter-sterilized (0.22 μm) and autoclaved at 121°C and 15 psi for 30 min. Subsequent transfers of 0.5 mL of these dilutions to 4.5-mL volumes of sterile water with 2,4-DCP yielded slurry dilutions with 10 mg/L 2,4-DCP per tube. Triplicate tubes were prepared at each dilution and incubated for 1 month before acetonitrile extracts of slurry dilutions were analyzed by a previously described HPLC protocol.[13] Tubes with a chromatogram having a 2,4-DCP peak area not more than three times the peak area of the 4-chlorophenol were scored positive. The most probable number of sediment dechlorinating microorganisms were estimated from the number of positive tubes in consecutive dilutions with an MS-DOS Turbo Pascal program using the American Society for Microbiology guidelines for MPN determinations.[20]

Analysis of variance, Duncan's multiple range tests, and regression analyses of data were performed using SAS (Release 6.03, SAS Institute, Inc., Cary, NC).

RESULTS AND DISCUSSION

Variability in DCP Dechlorination in Sediments

The time required for reductive dechlorination of 2,4-, 2,5-, and 3,4-DCP to monochlorophenols varied temporally and spatially in Cherokee sediments collected every other month between May 1988 and May 1989. Data for sites 1, 3, and 4 are summarized in Table 13.2. Microorganisms in sediments from these sites exhibited low (site 1), high (site 4), and intermediate (site 3) dechlorinating activity. Anaerobic dechlorinating microorganisms in sediments from sites 2 and 5 exhibited activities similar to those determined in site 3.

No seasonal pattern for dechlorination of the three isomers was evident from the data. With the exception of T_{50} values for the DCPs in July and November 1988 sediments (and in some January 1989 sediments), mean T_{50}

Table 13.2. Persistence of 2,4–, 2,5–, and 3,4–Dichlorophenol in Cherokee Sediment Slurries

Sampling Date	T_{50} (days)								
	Site 1			Site 3			Site 4		
	2,4–	2,5–	3,4–	2,4–	2,5–	3,4–	2,4–	2,5–	3,4–
May	24	26	25	21	20	32	21	22	25
July	192	173	160	88	98	93	12	17	19
September	72	17	29	11	15	20	12	18	20
November	91	155	159	25	92	139	19	14	21
January	10	14	66	7	15	64	10	14	20
March	43	41	50	7	7	6	7	7	9
May	19	16	32	16	21	35	12	9	45

Note: Numbers represent the mean time (n = 3) for dechlorination of 50% of the respective dichlorophenol to a monochlorophenol in three different sediments from the same site.

values for each of the isomers ranged from 6 to 30 days in sediments collected from all sites. Mean T_{50} values for DCPs in sediments from each site except site 4 were elevated in July and November.

Significant differences in the T_{50} values of the three DCPs were observed in sediments from different sites. The mean T_{50} value for each isomer was highest in sediments from site 1, averaging 64, 63, and 74 days for the 2,4-, 2,5-, and 3,4-isomers, respectively. Conversely, the mean T_{50} for each isomer was lowest in sediments from site 4. The 2,4- and 2,5-DCPs were dechlorinated more rapidly (T_{50} = 13 and 14 days, respectively) than 3,4-DCP (T_{50} = 23 days) in sediments from this site. A similar susceptibility to dechlorination was observed for 2,4-, 2,5-, and 3,4-DCP in sediments from site 3, with mean T_{50} values of 25, 38, and 56 days, respectively. Thus, a range of T_{50} values was observed for each isomer in Cherokee sediments from different sites.

In considering the influence of chlorine substitution on DCP dechlorination, a regiospecific pattern was observed for the initial dechlorination of each isomer. As was previously observed in the study of five pond sediments, the 2,4- and 2,5-isomers were reductively dechlorinated to 4- and 3-chlorophenol (CP), respectively, by removal of the *ortho* chlorine, and the 3,4-DCP was dechlorinated at the *para* position to form 3-CP. Dechlorination at the *meta* position of 3,4-DCP was detected only once in the 105 sediment slurries examined, and in this case, 3-CP and 4-CP were produced in approximately equal amounts.

Sediment/Water Characteristics

In order to determine any correlation between the T_{50} values for the DCPs and properties of the sediment-water system, the Cherokee sediment and water samples were analyzed for several physical, chemical, and microbiological characteristics. These included in situ temperature, pH, redox potential (E_h), sediment organic carbon, dissolved organic carbon, nitrate concentration, sulfate concentration, and the number of 2,4-DCP dechlorinating microorga-

Table 13.3. Sediment pH and Sulfate and Nitrate Concentrations (ppm) in Water from Three Sites in Cherokee Pond

Collection Date	Site 1			Site 3			Site 4		
	pH	SO_4	NO_3	pH	SO_4	NO_3	pH	SO_4	NO_3
May	6.4–6.6	0.23	ND[a]	6.3–6.6	0.17	ND	6.4–6.6	0.20	ND
July	6.3–6.7	0.36	ND	6.4–6.7	0.19	ND	6.4–6.5	0.58	ND
Sept.	5.8–6.1	0.77	0.73	6.0–6.1	1.11	0.71	6.0–6.2	0.76	0.72
Nov.	6.5–6.8	0.60	1.47	6.2–6.6	0.62	1.54	6.0–6.2	0.53	1.49
Jan.	5.9–6.0	0.37	ND	6.0–7.0	0.90	ND	6.5	0.08	ND
Mar.	5.8	NW[b]	NW	7.0	NW	NW	6.7	NW	NW
May	4.7–5.4	12.48	ND	5.1–5.7	11.73	ND	5.4–6.6	0.36	ND

Note: pH numbers represent the range of pH determined for three sediments from each site. Reported sulfate and nitrate concentrations are the mean of triplicate determinations of water samples from each of three locations within each site.

[a] Not detectable.

[b] No water column (pond drained).

nisms. Tables 13.3 and 13.4 summarize data on sediment pH, sulfate and nitrate concentration in site water, and number of 2,4-DCP dechlorinating sediment microorganisms of samples from sites 1, 3, and 4.

Some sediment/water characteristics displayed no significant temporal and/or spatial variation. Sediment temperatures ranged from 10 to 30°C during the sampling year but did not vary from one site to another by more than 0.5°C at any one sampling time. Similarly, water temperatures, which paralleled those of sediments from the same site, varied significantly temporally but not spatially. Mean sediment redox potentials fluctuated during the study period from –125 mV (May 1988) to –4 mV (September 1988). However, spatial variability was limited, as evidenced by the mean E_h range of –32 to –59 mV for sediments from all sites. Sediment redox potentials were highest (+130 mV) in May 1989, when the water column was restored after a 2-month period during which the pond was drained.

Sediment/water characteristics that displayed significant temporal and/or spatial variation were pH, organic carbon content, nitrate and sulfate concentration, and the number of 2,4-DCP dechlorinating microorganisms. The mean pH of sediments from all sites ranged from 5.3 to 7.1 during the study period. Extremely acidic pHs (i.e., 4.7) were reported for May 1989 sediments, which were collected after the water column was restored to the drained pond (Table 13.3). The organic carbon content of Cherokee sediments and water

Table 13.4. Most Probable Number (MPN) of 2,4-Dichlorophenol Dechlorinating Microorganisms in Cherokee Sediments

Sediment Collection Date	MPNs ($\times 10^3$/g dry sediment weight)		
	Site 1	Site 3	Site 4
September	0.18	0.52	33.00
January	4.60	<2.90	4.60
March	<8.50	19.00	48.00
May	<2.90	48.00	<2.90

varied to a greater extent temporally than spatially (data not shown). During the study year, sediment organic carbon averaged 0.1 to 2.0%. Mean concentrations of total organic carbon (TOC) for sediments from all sites ($n = 15$) were highest in September 1988 (0.68%), November 1988 (0.92%), and March 1989 (1.1%). However, in sediments from each of the individual sites ($n = 3$), average TOC was not necessarily elevated at these times. Mean TOC was highest in site 4 sediments (0.89%) and lowest in site 1 sediments (0.48%). Dissolved organic carbon, which was often less than 10 ppm, also varied significantly over time but not with site (data not shown).

The concentration of nitrate and sulfate in site water varied significantly over time and, in some instances, with site (Table 13.3). From September to November 1988, nitrate levels doubled from a mean of 0.72 ppm to a mean of 1.51 ppm. However, nitrate concentrations were not significantly different from site to site. With the exception of September 1988 and May 1989, at every sampling time the average sulfate concentration in water from each site was less than 1 ppm. During the year, sulfate concentrations increased in water samples collected from May through September but generally decreased in water samples collected from September through January. The highest concentrations of sulfate, which occurred in the May water samples from sites 1 (12.48 ppm) and 2 (11.73 ppm), coincided with the restoration of the water column following drainage of the pond. Why a similar peak in sulfate concentration was not observed in water from site 4 is not clear. However, over the study period, the mean sulfate concentration (0.42 ppm) was lowest in water from this site.

The number of 2,4-DCP dechlorinating microorganisms in Cherokee sediments varied temporally and spatially (Table 13.4). At any one sampling time (except January 1989) or site, the number of these organisms in sediments varied from one to two orders of magnitude. MPN values of dechlorinating microorganisms in most sediments increased from September to January and from January to March, and decreased from March to May. Drainage of the pond may have affected MPN values of dechlorinating microorganisms in sediments collected in May.

Correlation of DCP Dechlorination to Sediment/Water Characteristics

A statistical analysis of the data indicated that certain sediment characteristics may be used to predict the T_{50} values of selected DCPs. For each of the isomers, a multiple regression analysis was performed using data from all sites and sampling dates. However, for 2,4-DCP, regression analysis indicated that none of the variables (alone or in combination) accounted for more than approximately 50% of the variation in the T_{50} values. Thus, additional research is needed to identify other sediment characteristics useful in predicting T_{50} values of 2,4-DCP and to determine the reasons that this isomer differs from the others.

Although the number of dechlorinating microorganisms present in the sedi-

Table 13.5. Summary of Stepwise Regression for Dependent Variable T_{50}

Step	Variable Entered	Number In	Partial R^2	Model R^2	F	Prob>F
1	pH	1	0.5375	0.5375	39.5086	0.0001
2	NO_3	2	0.1946	0.7321	23.9701	0.0001
3	SO_4	3	0.0718	0.8039	11.7246	0.0017
4	Eh	4	0.0252	0.8291	4.5726	0.0405
5	CO_2/H_2	5	0.0183	0.8474	3.5931	0.0677
6	TOC	6	0.0154	0.8628	3.2658	0.0811

	DF	Sum of Squares	Mean Square	F	Prob>F
Regression	6	126835.3518	21139.2253	30.41	0.0001
Error	29	20160.9538	695.2053		
Total	35	146996.3056			

Variable	Parameter Estimate	Standard Error	Partial Sum of Squares	F	Prob>F
Intercept	–605.1028	149.7888	11345.2115	16.32	0.0004
pH	115.6724	25.2056	14641.2417	21.06	0.0001
NO_3	105.3080	19.0325	21283.4639	30.61	0.0001
SO_4	–136.4994	30.1955	14206.5686	20.44	0.0001
E_h	0.7187	0.2625	5210.0869	7.49	0.0105
CO_2/H_2	0.0400	0.0184	3300.1914	4.75	0.0376
TOC	– 0.0050	0.0027	2270.3916	3.27	0.0811

ments at the time of 2,4-DCP addition could be not used to predict T_{50} values of this isomer, ongoing experiments in our laboratory indicate that a "threshold" number of actively dechlorinating microorganisms may be necessary before loss of the parent compound is detectable. The adaptation period (i.e., time during which no dechlorination is detectable) of a T_{50} value may thus include an induction-followed-by growth response, as proposed by Linkfield et al. in their study of the adaptation (acclimation) period before dehalogenation of halobenzoates.[21] Recent work by Cole and Tiedje[22] and by DeWeerd and Suflita[23] with the halobenzoate dehalogenating microbe *"Desulfomonile tiedjei"* indicates that the dehalogenating activity of this organism is inducible. Dolfing and Tiedje[24] have also reported a growth yield increase from the reductive dechlorination of 3-chlorobenzoate in a methanogenic coculture with this microorganism. The dechlorinating microorganisms in Cherokee sediments may be similar to this well-characterized organism[25,26] with respect to the inducibility of dechlorinating activity and increased growth from the reaction. If this is the case, a better understanding of the factors governing the induction-followed-by-growth response should improve the prediction of T_{50} values.

Regression analysis of data for both 2,5- and 3,4-DCP indicated that the variables of pH, nitrate and sulfate concentrations, and redox potential accounted for 83% of the variation in their T_{50} values (Table 13.5). Approximately 86% of the variation in T_{50} values of these isomers was explained by using these variables in addition to the ratio of carbon dioxide to hydrogen in the headspace of the test vessels (data not shown) and sediment total organic carbon (TOC). The variables of pH, nitrate, redox potential, and the ratio of carbon dioxide to hydrogen in serum bottle headspaces were positively correlated to T_{50} values; sulfate and TOC were negatively correlated to these values. The negative correlation of sulfate to T_{50} values may result from the enhanced activity in the presence of sulfate of a dechlorinating organism similar to the sulfidogenic *"Desulfomonile tiedjei."* However, this finding contrasts to published results of other researchers on the inhibitory aspects of sulfate on reductive dechlorination.[8,12,27] Thus, further studies should determine the utility of the model in predicting the T_{50} values of DCPs in sediments from other ponds, such as those in the initial study, with different sulfate regimes and sediment/water characteristics.

CONCLUSIONS

The results of the present study indicate that the reductive dechlorination of chlorinated phenols in anaerobic sediments can be correlated to sediment/water properties. In particular, the pH, nitrate and sulfate concentrations, and E_h of sediment-water systems may be used to predict the persistence of selected DCPs, with chlorines removed from the ring in the order *ortho* > *meta* or *para*. The use of these variables has practical implications because they can be determined rapidly with pH, ion selective, and E_h electrodes. Levels of the anions may also be determined by ion chromatography. The data may then be used in regression equations to predict T_{50} values of specified chlorophenols.

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DISCLAIMER

Mention of trade names or commercial products does not constitute endorsement or recommendation for use by the U.S. Environmental Protection Agency.

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B. Cometabolism

CHAPTER 14

The Effects of Groundwater Chemistry on Cometabolism of Chlorinated Solvents by Methanotrophic Bacteria

Anthony V. Palumbo, William Eng, and Gerald W. Strandberg

INTRODUCTION

Degradation of chlorinated alkenes such as trichloroethylene (TCE) by methanotrophic bacteria is a promising technology for the remediation of contaminated groundwater.[1] Ultimately, the success of this approach may be dependent on the influence of groundwater chemistry on degradation rates and extent. TCE can rapidly be reduced to low levels in laboratory cultures growing on defined media.[2] However, if major changes in groundwater chemistry are necessary to achieve substantial TCE degradation, field application of processes (i.e., above ground and in situ treatment) may be limited by cost or logistic problems. For example, the presence of competitive inhibitors may limit the extent of TCE degradation.

Biodegradation of TCE by a variety of mechanisms has been reported.[1,3-10] Some investigators have reported anaerobic degradation of TCE,[8,11] which can apparently produce dichloroethylene (DCE) and vinyl chloride,[12] a known carcinogen, and at contaminated sites TCE is often found in association with DCE. Degradation of TCE to CO_2 by aerobic mixed cultures has also been reported, but the degradation mechanisms have not always been clearly identified.[3,4] TCE degradation by methanotrophs is apparently initiated by the methane monooxygenase (MMO) using a cometabolic process. Normally, methane is oxidized to methanol by MMO.[13] But apparently TCE is also fortuitously oxidized by the monooxygenase. TCE breakdown may begin with epoxidation of the double bond, eventually resulting in formation of carbon dioxide, glyoxylic acid, and dichloroacetic acid, while a small fraction of the carbon from the TCE can be incorporated into the cells.[7] MMO is a fairly nonspecific enzyme and oxidizes methane, ammonia, TCE, and many other compounds.[13] Competitive inhibition may limit the degradation of TCE in the presence of high levels of methane and ammonia; thus, high rates of methane addition to a bioreactor or high levels of ammonia in the groundwater could limit TCE degradation. Another potential effect of groundwater chemistry is modification of the form or specificity of methane monooxygenase. Methane monooxygenase can exist either in free or particulate form depending on culture conditions, and the two forms can exhibit different rates of TCE degradation.[2,9]

The goal of our research is to quantify the potential effects of groundwater chemistry on the biodegradation of TCE by methanotrophs and to define concentrations of methane that need to be added to the system to produce maximum rates of TCE degradation. This includes evaluation of major nutritional requirements (e.g., PO_4) in addition to the focus on competitive inhibition.

MATERIALS AND METHODS

Cultures and Culture Conditions

We examined the degradation of TCE in batch experiments using *Methylosinus trichosporium,* strain OB3b; an isolate (46–1) previously obtained from an Oak Ridge Site;[7] and mixed methylotrophic cultures (JS, S1, DT1, and DT2) isolated from TCE-contaminated sites. *Methylosinus trichosporium* (strain OB3b) was provided by M. E. Lidstrom (California Institute of Technology). The JS mixed culture was isolated from a waste disposal site in Oak Ridge[7] and is currently being used in bioreactor studies of TCE degradation.[14] The DT1 and DT2 mixed cultures are bacteria-amoeba consortia isolated from an Oak Ridge site (R. L. Tyndall, Oak Ridge National Laboratory, personal communication). The S1 consortia was obtained during this study from contaminated groundwater from a Kansas City Department of Energy (DOE) site.[15]

Two types of batch experiments were run with the cultures. In the first type both degradation and growth were observed in 100 mL of sterile mineral salts medium (NATE),[11] prepared in 250 mL culture bottles, and inoculated with 1 mL of starter cultures. The medium contained 50 μg/L $CuSO_4{\cdot}5H_2O$, 10 μg/L $MnSO_4{\cdot}H_2O$, 70 μg/L $Zn(NO_3)_2{\cdot}6H_2O$, 10 μg/L $CoCl_2{\cdot}H_2O$, 10 μg/L MoO_3, 1 g/L $MgSO_4{\cdot}7H_2O$, 0.2 g/L $CaCl_2$, 1 g/L KNO_3, 0.1 g/L NH_4Cl, 10 mL of 0.27 g/L $FeCl_3$, and 20 mL of 5% potassium phosphate buffer (pH 6.8). Final media pH was adjusted to 6.8. The headspace of each bottle contained 8–8.5% (v/v) methane and in air except as otherwise noted. Each bottle was sealed with a Teflon septum, and to ensure an airtight seal, modeling compound, sandwiched between parafilm, was used to cover the bottle caps. The culture bottles were shaken (inverted, to further guard against gas leakage) on a rotary shaker (fermentation design) at 75 rpm. TCE, oxygen, and methane concentrations in the headspace were followed over time by periodic sampling of 0.5 mL of the headspace with a gas syringe (Hamilton). This design allowed for observation of the growth, methane consumption, and oxygen consumption by the cultures under various conditions. However, because initial populations of bacteria were low, degradation rates were also relatively low.

In the second series of batch experiments, strain OB3b was inoculated at higher cell densities to achieve higher rates of TCE degradation. The bacteria were cultured in 250 mL bottles containing NATE medium and 20% (v/v) methane in the headspace at 22°C for 5 days on a shaker table at 75 rpm. After incubation the cells were concentrated by centrifugation at 2500 rpm for 45 min at 10°C. Harvested cells were then resuspended in a 5 mM phosphate buffer at pH 7.0. Aliquots (12 mL) of the concentrated culture were added to 50-mL vials containing NATE to yield a final concentration of 0.02–0.04 mg/mL of cell protein.

Degradation of TCE was followed in small (50-mL) vials sealed with Teflon-lined septa; three vials were used for each treatment. In most experiments the concentration of one of the components of the media (i.e., ammonia) was varied to generate the different treatments. All vials contained 10% (v/v) methane in the headspace unless otherwise noted and were placed on a shaker table at 75 rpm. A TCE-saturated water solution was added by syringe injection, yielding a nominal initial TCE concentration of 3.0 ppm. At 24-hr intervals over a 3- to 4-day period, bottles from each treatment were sacrificed by addition of 4 mL of hexane to stop the biological activity and extract the remaining TCE. Vials remained on the shaker table for at least 24 hr before GC analysis. Over the range of concentrations tested, the hexane extraction removed > 95% of the TCE added.

Additional experiments were conducted in a bench-scale continuous-flow bioreactor[14] consisting of a 5-cm i.d. × 110-cm long glass column packed with 0.6-cm ceramic berl saddles as a support for the biofilm. The system was a trickle-type packed-bed bioreactor with a gas stream (25 mL/min) containing 4% (v/v) methane and air introduced at the top of the bioreactor. The bioreactor was inoculated with pink-tinted mixed culture (JS) containing methanotrophs. Phospholipid analysis indicated that Type I methanotrophs predominate in the culture (D. C. White, personal communication). With the same mineral salts media used in the batch cultures, 1 mg/L TCE influent concentration, and a 50-min residence time, approximately 50% of the TCE was degraded in a single pass through the bioreactor; further degradation of TCE is evident with liquid recycle.[15]

Analytical Methods

Protein concentration was determined using the Coomassie Blue dye-binding method.[16] A Bio-Rad protein assay kit was adapted for use with a centrifugal fast analyzer (COBA-FARA) and used for the measurements.[17]

TCE concentration in the hexane extract from experiments with the concentrated cell suspensions and in the headspace gas in the other experiments was determined using a Perkin Elmer Sigma 2000 GC with electron capture detector and a 3-ft × 1/8-in. glass column containing 60/80 1% Carbopack B. Other GC operating parameters were

- 100°C oven temperature
- 125°C injector temperature
- 350°C detector temperature
- 30 mL/min of N_2 carrier gas

With these settings TCE retention time was 5.2 min. Analysis of *trans*-1,2-DCE was done using the same gas chromatography parameters as described for TCE analysis except oven temperature was set at 65°C. This gave a DCE retention time of 2.05 min. Liquid *trans*-1,2-DCE standards were used to calibrate the gas chromatograph daily.

In some experiments, ^{14}C-labeled TCE was used to determine TCE degradation rates and breakdown products.[7] After incubation with [1,2-^{14}C] TCE (3.0 mCi/mmol [111 MBq/mmol], Pathfinder Laboratories, St. Louis, MO), the pH of the media was adjusted to 9.5–10, thereby converting all CO_2 gas into carbonate. A subsample of culture was centrifuged, and the pellet was resuspended in NATE to assess the amount of TCE incorporated into cellular material. Remaining TCE was extracted from the supernatant with hexane. Acid was added to convert the carbonate into CO_2, which was trapped in 0.1 N NaOH in a small vial placed in the culture bottle. Subsamples of the water phase and the trapped CO_2 were then counted using a TriCarb 2000CA liquid scintillation analyzer, (Packard, Downers Grove, IL).

Oxygen and methane concentrations in headspace gas were measured with a Perkin Elmer 3920B gas chromatograph equipped with a 6 × 1/8 in. molecular sieve 5A column (Supleco) and a thermal conductivity detector. Both the injector and interface temperature were set at 150°C. Oven temperature (initial and final) was set at 45°C. Retention times were 0.68, 1.3, and 2.3 min for oxygen, nitrogen, and methane, respectively. Oxygen and methane concentrations are reported as percent (v/v) of headspace gas.

Experiments

The effect of ammonia on TCE degradation was examined using concentrated OB3b cells and with a nonconcentrated mixed culture (DT). The experiment with the OB3b cells was conducted in 50-mL vials as described above in NATE media with modified ammonium chloride concentrations of 0 to 1.0 mg/L, and TCE degradation was followed by GC techniques. Growth of the DT culture was followed in the 250-mL bottles as previously described, and the fate of TCE was followed using radiolabel techniques at concentrations of 0.1 and 2.5 mg/L of ammonia chloride. In the experiment with the DT culture, initial methane and oxygen were 8 and 18% of headspace gas, respectively.

The effect of phosphorus concentration on growth and TCE degradation was determined in an experiment where five concentrations of phosphorus (1.4, 3.2, 6.2, 25, 100 μg/mL) were used. A total of 29.6 μg (final concentration = 0.29 μg/mL) of TCE was added to each bottle. Nonconcentrated cells of strain 46–1 were used in these experiments.

The growth of the JS mixed culture was examined at various methane levels after adding NATE elements (to make up 10% of the final volume) to the site water (90% of final volume). Methane levels were 1.2, 3.5, 5.5, 7.5, and 9.7% (v/v) of headspace. Initial oxygen levels were 18% (v/v) of headspace for all treatments. Initial concentration of TCE in the incubation water was about 4.7 mg/L.

In a second experiment using site groundwater, the proportion of groundwater was varied by the addition of NATE nutrients and distilled water to yield final concentrations of site water of 22.5, 45, 67.5, and 90%. The purpose of this experiment was to determine the effect of TCE concentration and micronutrient conditions on the growth and TCE degradation. We determined that the site groundwater initially contained 12.3 mg/L and 4.7 mg/L TCE, and the concentrations in the treatments varied in proportion to the amount of site water added. Initial methane and oxygen concentrations were 8 and 18% (v/v) of headspace gas, respectively.

The presence of the DCE in the site water prompted us to examine the degradation of TCE in the presence of *trans*-1,2 DCE. A comparison of the ability of the DT2 and JS consortia to degrade TCE (0.3 mg/L) with 0, 30, and 63 mg/L of *trans*-1,2-DCE added to the bottles was conducted to determine if DCE inhibited TCE degradation. Initial methane and oxygen concentrations were 8 and 18% (v/v) of headspace gas, respectively. TCE degradation was followed using both radiolabel and GC techniques, and degradation was followed using GC analysis.

The effects of methane (0 to 20% v/v), ammonia (0, 2.5, and 10 mg/L), and copper (0.2 and 1.0 mg/L) concentrations on TCE degradation were examined in the fixed-film, packed-bed bioreactor system. TCE degradation was compared among the different treatments by analysis of the offgas.

Data Analysis

Analysis of variance (ANOVA) with Duncan's multiple range test was used to test for significant treatment differences in total TCE transformation and the transformation to breakdown products in experiments using the radiolabeled TCE. Other data were analyzed using Lotus 1–2-3 (Lotus Corporation)

RESULTS AND DISCUSSION

Growth of Cultures Exposed to Site Water

Although at all concentrations of site water the JS consortia eventually consumed the same amount of methane and oxygen, consortia growing in the higher concentrations of the site water consumed the methane and oxygen at a slower rate, indicating a decreased growth rate (Figure 14.1a). The unsterilized treatment without any inoculum contained 25% site water and also displayed methane and oxygen consumption, indicating the presence of methane-utilizing organisms in the site water. The rate of methane and oxygen consumption, however, was not as rapid as in the treatment containing 25% site water with added JS mixed culture (Figure 14.1).

Although an enrichment (S1) from the site water was used in subsequent

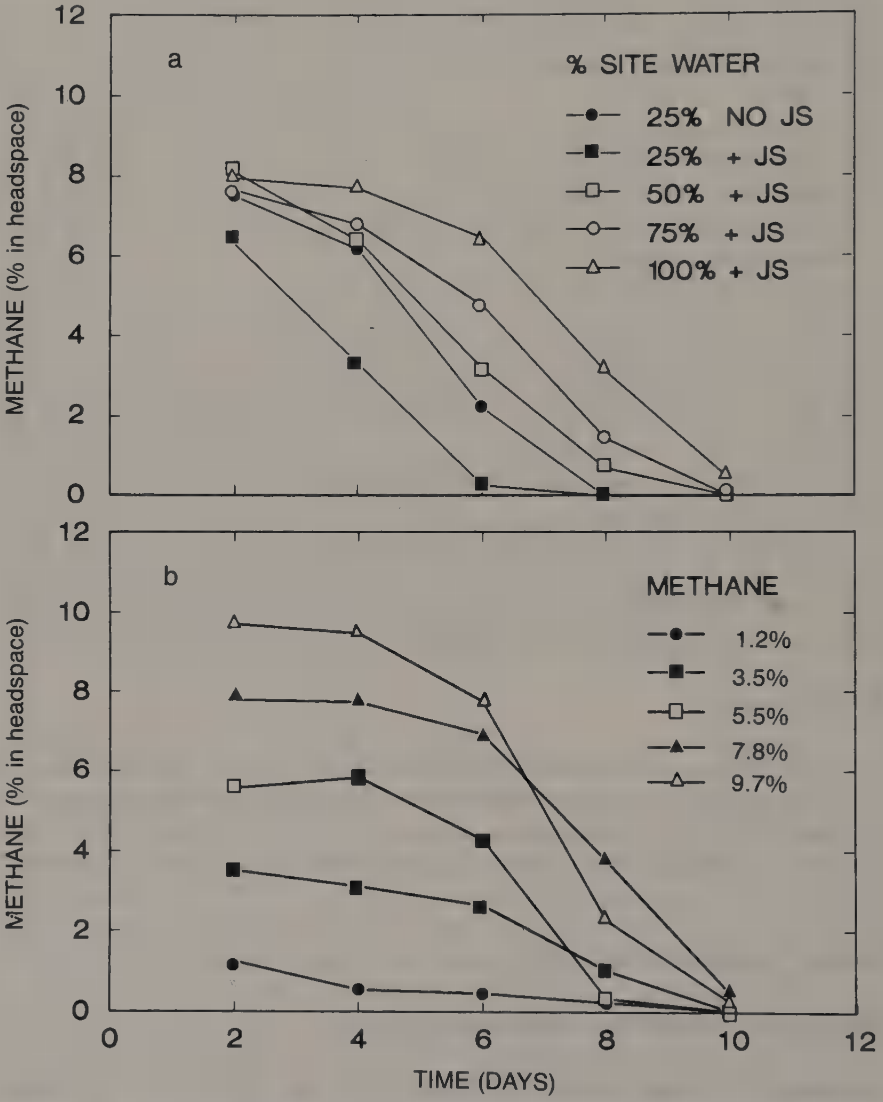

Figure 14.1. Methane utilization by the JS consortia growing in site water: *(a)* with varying percentages of site water; *(b)* with varying initial methane concentrations.

experiments and was demonstrated to degrade TCE, no evidence for degradation of TCE was observed in these experiments with site water with the added JS culture. The lack of significant TCE degradation despite the evidence for growth of the methanotrophs (utilization of methane) indicates that there was inhibition of the TCE degradation in the site water.

In the second experiment, examining the effect of methane on the growth of methane-utilizing consortia exposed to the site water, the methane was substantially depleted in all treatments after 10 days (Figure 14.1b). As in other experiments, oxygen consumption was significantly correlated with the amount of methane consumed ($r^2 = 0.995$, $N = 5$), and there was no evidence for TCE degradation.

Much of the impetus for the following work came from the observation that in the water from the DOE Kansas City site we could demonstrate growth of methane-utilizing bacteria (Figure 14.1), but there was no evidence for TCE degradation in the site water.

Biodegradation of TCE in the Presence of *trans*-1,2-DCE

The results of the DCE experiments indicated that DCE could have had an effect on the TCE degradation rates in the site water. Both the DT2 and JS consortia transformed significantly ($F = 19.88$; d.f. = 2, 13) greater amounts of TCE at lower *trans*-1,2-DCE concentrations (Figure 14.2). The decrease in the extent of degradation of the TCE was statistically significant (95% level) and was proportional to the concentration of *trans*-1,2-DCE added; the correlation coefficient (r) for the relationship between the percent degradation of TCE, measured using the radiolabel data, and the *trans*-1,2-DCE concentration was -0.92 ($n = 6$) for the DT2 cultures and –0.94 ($n = 6$) for the JS cultures. The DT2 culture was significantly ($F = 12.54$; d.f. = 1, 11) more efficient at degrading the TCE, transforming a mean of 22.27% of the added TCE compared to a mean of 12.38% transformation by the JS mixed culture. The GC data gave similar results for total TCE conversion (data not shown). Both consortia converted a high percentage of the TCE to CO_2, and there was no significant difference ($F = 1.73$; d.f. = 1, 11) between the consortia in the proportion of the transformed TCE that was converted to CO_2 (mean = 50.5%).

DCE degradation in the bioreactor was more efficient than the TCE degradation.[14] In a single pass the bioreactor removed ~ 50% of a 1 ppm solution of TCE but reduced a 1 ppm solution of *trans*-1,2-DCE to below the limit of detection. At these equal concentrations of TCE and DCE there was no obvious effect of *trans*-1,2-DCE on TCE degradation.[14] DCE degradation by methane-utilizing cultures is well documented.[9,18] However, demonstration of the competitive inhibition between TCE and DCE degradation is less well documented but could be important due to their co-occurrence. The kinetics of the inhibition are currently the subject of more detailed study in our laboratory. Because the laboratory doing the analysis at the site reported that the

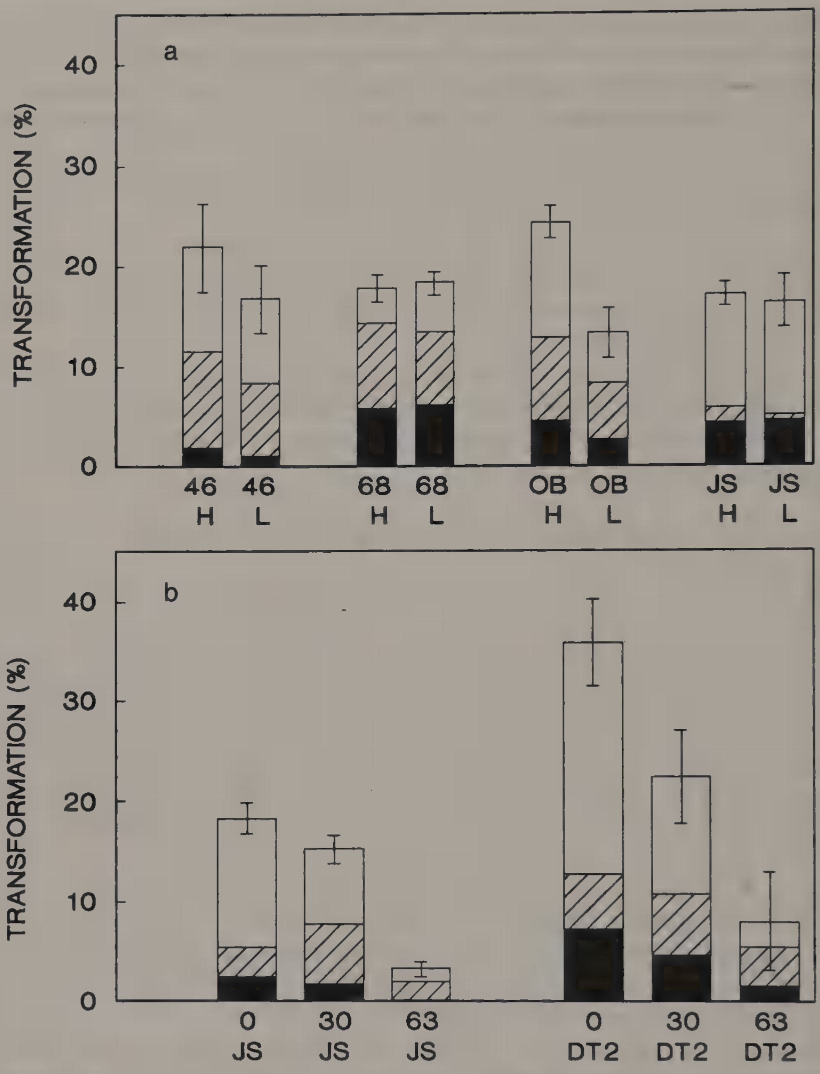

Figure 14.2. Distribution of ^{14}C label as percent of added TCE (0.3 mg/L): *(a)* comparison of distribution by the 46–1, 68–1, and OB3b cultures, and the JS consortia; *(b)* comparison of JS and DT2 consortia with 0, 30, and 63 mg/L added *trans*-1,2-DCE. The amount incorporated into cell material is given by the *solid bars* on the bottom, into water soluble products is given by the *cross-hatched bars* in the center, and into CO_2 is given by the *open bars* on the top. The range for the total transformation is given by the error bars.

DCE present was in the *trans* form, *trans*-1,2-DCE was used in these experiments. However, the laboratory is now reporting that the DCE is present in the *cis* form. This should not affect the conclusions since other experiments have indicated that the *cis* form may have a more pronounced effect on the TCE degradation rate (Eng, unpublished data).

Effects of Ammonia, Methane, and Phosphorus on TCE Degradation

In the batch experiments high concentrations of added ammonium chloride appeared to inhibit TCE degradation by OB3b. Cultures incubated in the presence of 0.05 mg/L ammonium chloride had higher initial rates (1.35 μg TCE/hr/mg protein) of TCE degradation than cultures without ammonium or with higher concentrations of ammonium (Figure 14.3). In an experiment conducted with a mixed culture with nonconcentrated cells, increasing the ammonia concentration from 0.1 to 2.5 mg/L resulted in a 50% decrease in amount of ^{14}C TCE degraded during the growth of the bacteria. The proportion of $^{14}CO_2$, water-soluble products, and cell-bound ^{14}C did not vary at different ammonia concentrations. In the absence of methane, degradation was not detected ($< 3\%$) regardless of ammonium concentrations (Figure 14.4).

Methane was similarly demonstrated to reduce TCE degradation in both pure and mixed culture studies. In experiments with 0, 2, 10, and 20% (v/v) methane in the headspace of the vials, maximum degradation (>3.0 μg TCE/hr/mg protein) by the concentrated OB3b cultures was observed in the treatments with only 10% (v/v) methane in the headspace. In the absence of

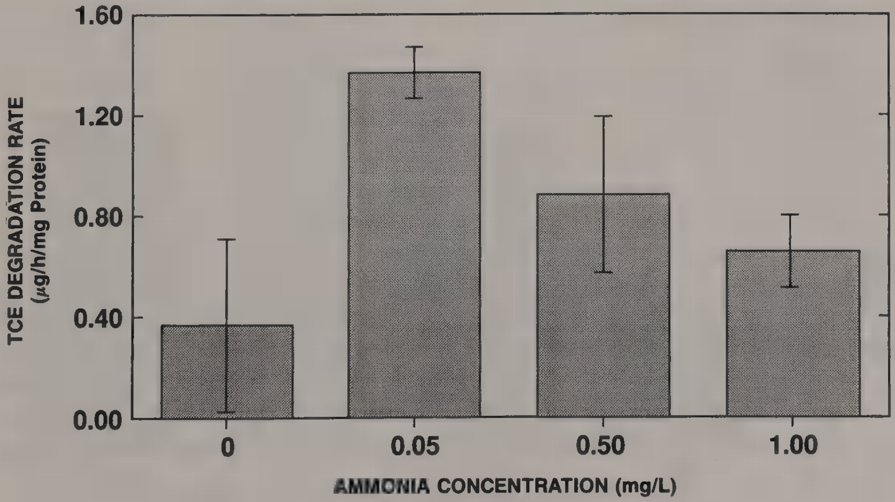

Figure 14.3. TCE degradation rate (μg/hr/mg protein) by OB3b with increasing concentrations of ammonium chloride. Data are given as calculated slope ± SE.

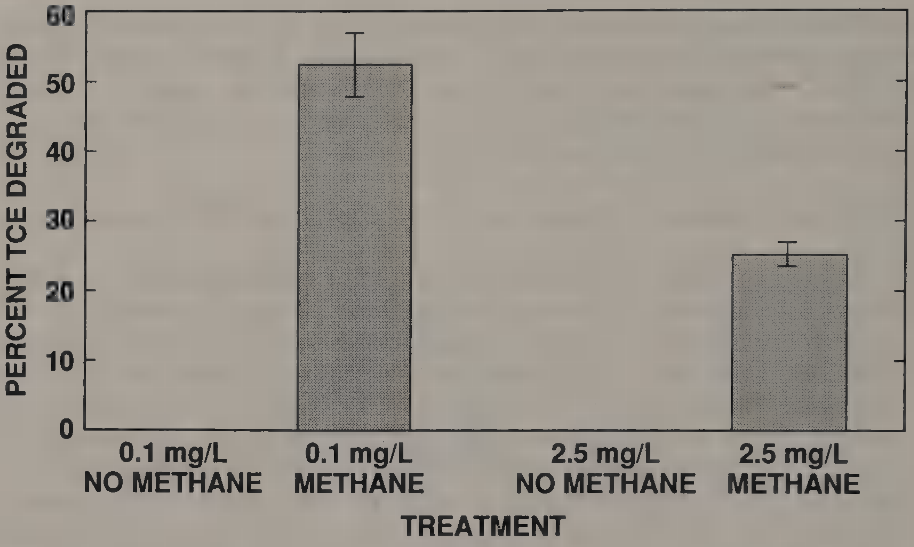

Figure 14.4. TCE degradation (%) by mixed culture DT1 at 0.1 and 5.5 mg/L ammonium chloride with and without methane.

methane in the headspace, degradation was substantially lower (0.40 μg TCE/hr/mg protein). The rate of TCE degradation progressively declined at a concentration of 20% (v/v) methane in the headspace (Figure 14.5). In the mixed culture experiments the effect of methane on TCE degradation rate was apparent at relatively high levels of methane. We found that 29% of the added TCE

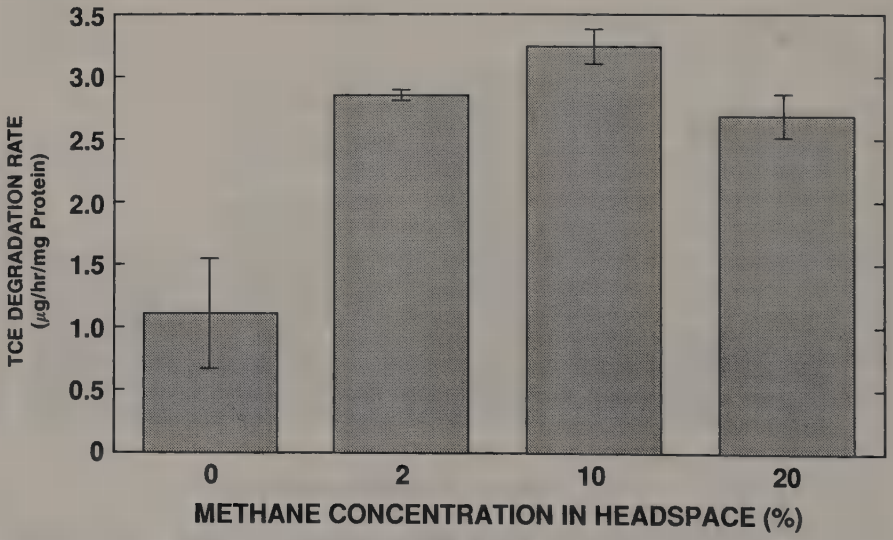

Figure 14.5. TCE degradation (μg/hr/mg protein) by OB3b with increasing concentrations of methane.

was transformed by strain 46-1 with 10% added methane (10% to CO_2), and only 16% of the TCE was transformed with 16.7% methane (6% to CO_2). The decrease in degradation was probably due to competitive inhibition.

Methane concentrations at or above 4% (v/v) supported methanotrophic growth, and TCE and *trans*-1,2-DCE degradation in the bioreactor. A methane concentration of 2% was not sufficient to support the predominance of the methanotrophic population. Over time a yellow-pigmented, non–TCE-degrading species developed rapidly at 2% methane. In shorter experiments, cutoff of the methane supply resulted in a slight decrease in degradation over 4 hr, followed by a rapid decrease and a complete halt in TCE degradation by 14–16 hr. A resumption in the methane supply resulted in resumption of TCE degradation within 1–2 hr. Methane concentrations of 10% could not be shown to significantly reduce the extent of TCE degradation. However, the sensitivity of TCE degradation rates in a bioreactor to methane has been shown by Leahy et al.[6]

There is considerable evidence for ammonia oxidation by methanotrophs, and MMO is implicated in some of these studies.[13] The presence of ammonia can reduce the growth rate of methanotrophs,[11] presumably by competitive inhibition of methane uptake.[19] Thus, it is likely that the effect on TCE degradation rates is also competitive interactions since both TCE and methane are competing for sites on the MMO.

Variations in phosphorus content in the range tested affected the growth rate of the organism but not the rate of TCE degradation. The methane and oxygen consumption data indicate that the variations in phosphorus content slightly affected the growth rate of the organism (Figure 14.6), with higher levels of phosphorus leading to higher growth rates. However the rate or extent of TCE degradation was not significantly affected (data not shown). Thus, the concentrations of phosphorus in the media can be significantly reduced from those present in the original formulation of the NATE media, which contains phosphorus as a pH buffer as well as a nutrient. Since pH can be an important parameter in TCE degradation,[9] some buffering of pH may be needed.

Changes in media composition in the bioreactor did not result in changes in the extent of TCE degradation similar to those observed in the batch experiments. The effect of ammonia was not seen in an experiment where concentrations of ammonia chloride of 0, 2.5, and 10 mg/L were used. However, peak TCE degradation was seen in the batch experiments at a concentration only slightly greater than 0 (0.05 and 0.1 mg/L) ammonia chloride. Thus, concentrations between 0 and 2.5 mg/L may yield different results. Also, changes in copper and manganese concentration and the addition of 0.1 mM EDTA all had no effect on the TCE degradation rate. The general lack of effects in the bioreactor could be due to the design of the bioreactor masking effects that are evident in the batch systems. For example, the higher biomass may lead to copper limitation at higher copper levels than in the batch cultures. More work

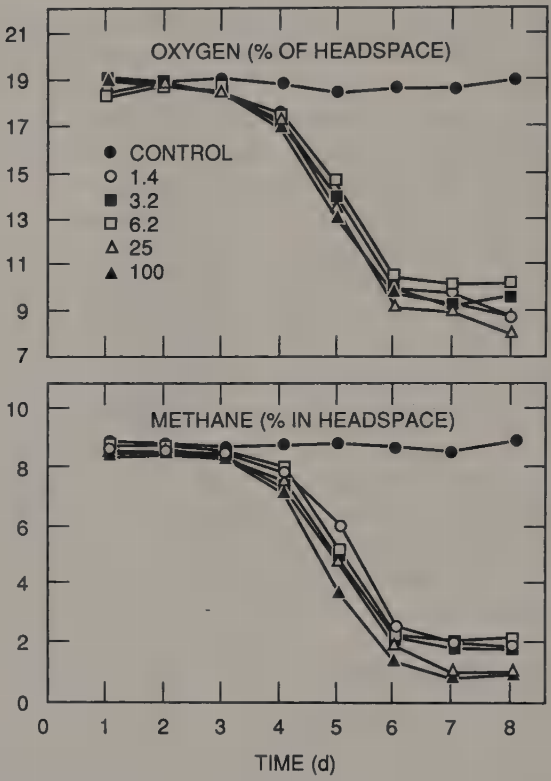

Figure 14.6. Methane and oxygen consumption by strain 46–1 at 1.4 (○), 3.2 (■), 6.2 (□), 25 (x), and 100 (▲) μg/mL of phosphorus and the control (●).

needs to be done examining the effects of factors that change TCE degradation rates in batch cultures in bioreactor systems.

Other experiments have indicated that manganese concentrations may influence degradation rates.[15] Manganese is a cofactor of some oxygenase enzymes,[4] and alterations of its concentration may affect the specificity of the methane monooxygenase. However, there may be a more subtle effect involved. In the defined media the presence of chelators results in changes in the concentration of the free form of all the trace metals when the total concentration of one of them is varied. Thus, in order to isolate effects to changes in the activities of one trace metal, experiments must be designed in which the activity of only one component varies. Thus, defining the specific trace metals involved in controlling the TCE degradation rate will be a complex undertaking. The influence of trace metals on TCE degradation could be due to shifting of methane monooxygenase between the soluble and the particulate form, as has been shown to occur under conditions of copper limitation.[2,9]

SUMMARY

Our results indicate that constituents of the medium have a significant effect on TCE degradation by methanotrophs. Depending on the initial composition, substantial modifications in the chemical composition of groundwater may be required to achieve rapid rates of TCE degradation in aboveground bioreactors. Ammonia concentration has been shown to be an important factor in these studies. In batch cultures ammonia clearly limits TCE degradation by both pure and mixed cultures. However, in bioreactors the effect is not clear and warrants further study. Phosphorus concentrations can clearly be substantially reduced from those present in the NATE without reducing degradation rate.

The lack of evident degradation of TCE in the Kansas City site samples may have been due in part to the presence of DCE in the site water. This phenomenon would probably be less important in bioreactors where the active microbial biomass would be much higher. Recent experiments have shown that DCE and TCE do degrade simultaneously and appear to be competitive inhibitors.[20] Ammonia has never been analyzed for at the site, but conditions are reducing so ammonia may have had an effect on decreasing the TCE degradation in the site water.

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CHAPTER 15

Anaerobic Degradation of Aromatic Hydrocarbons and Aerobic Degradation of Trichloroethylene by Subsurface Microorganisms

Dunja Grbic-Galic, Susan M. Henry, E. Michael Godsy, Elizabeth Edwards, and Kevin P. Mayer

INTRODUCTION

Homocyclic and heterocyclic aromatic hydrocarbons, which are constituents of petroleum, oil derivatives, and pesticide mixtures (e.g., creosote), and chlorinated aliphatic hydrocarbons, which are extensively used as solvents, are frequent subsurface contaminants.[1] Some of these compounds are known or suspected carcinogens; many of them are toxic. Their fate and transport in the subsurface environment will depend on their own physical and chemical characteristics and those of the environment and will be influenced by physical, chemical, and microbiological environmental processes. Among these, microbiological processes can change the pollutants and influence their fate the most profoundly. In numerous cases, microorganisms will cause complete degradation (mineralization) of the pollutant molecules, provided the environment is conducive to microbial growth and metabolism.

Homocyclic aromatic hydrocarbons are completely degraded by microorganisms under aerobic conditions, in the presence of molecular oxygen as a reactant and an electron acceptor for microbial respiration.[2] Homoaromatics are fairly reduced molecules and therefore prone to oxidative transformations. They are also quite stable resonant structures, but microbial oxygenases, which introduce atoms of oxygen into the aromatic molecule, are powerful enough catalysts to exert this transformation under physiological conditions. Aerobic microbial degradation of aromatic hydrocarbons is well understood, and aerobic microbial processes are widely used for *in situ* treatment of petroleum-contaminated groundwater aquifers.[1] However, microbial activity quickly depletes oxygen, and frequently the rate of oxygen consumption exceeds the rate at which oxygen can be introduced into a contaminated subsurface habitat. As a result, anaerobic conditions develop. These phenomena stress the importance of understanding the possibilities of anaerobic microbial transformation of aromatic hydrocarbons.

Heterocyclic aromatic hydrocarbons, which contain nitrogen or sulfur in their ring structure, are less stable than homoaromatics; therefore, they can be microbially oxidized not only using molecular oxygen but also using water as an oxygen source.[3] In some cases, it has been shown that the early oxidation products from these compounds are the same under aerobic and anaerobic conditions.[4–6] It would be logical to expect that these compounds will be easier to deal with anaerobically than their homocyclic counterparts.

Chlorinated aliphatic hydrocarbons (C_1 or C_2) with more than two chlorines per molecule are highly oxidized chemicals that are more prone to reductive than to oxidative transformations.[7] Under anaerobic conditions, these compounds are sequentially reduced to less chlorinated products: for example, tetrachloroethylene (PCE) and trichloroethylene (TCE) yield 1,2-dichloroethylene isomers (DCE) and vinyl chloride (VC);[8,9] carbon tetrachloride (CT) is frequently dehalogenated to chloroform (CF).[10] These processes naturally occur in contaminated groundwater aquifers.[11] Such transformations are not desirable, however, because the products can be more carcinogenic (e.g., VC) or toxic (e.g., CF) than the parent compounds. From this standpoint, it is necessary to understand the conditions that are conducive to, and the prerequisites for, aerobic transformation of halogenated solvents. As was first discovered by Wilson and Wilson in 1985,[12] some chlorinated solvents, such as TCE, can indeed be transformed not only anaerobically but also aerobically.

In the last several years, a lot of the work in the laboratories within the Environmental Engineering and Science Program at Stanford University has been devoted to studies of anaerobic transformation of homocyclic and heterocyclic aromatic hydrocarbons under methanogenic conditions and of aerobic transformation of TCE by methanotrophic populations and communities. Some of this work, specifically using groundwater aquifer microcosms, enrichments, and pure cultures, will be summarized in this chapter.

ANAEROBIC MICROBIAL TRANSFORMATION OF AROMATIC HYDROCARBONS

Background

The first experimental results indicating anaerobic microbial oxidation of homocyclic monoaromatic hydrocarbons were published in 1980.[13] The authors observed formation of small amounts of $^{14}CO_2$ and $^{14}CH_4$ from ^{14}C-labeled toluene and benzene in methanogenic microcosms derived from salt-marsh and estuarine sediments contaminated by petroleum. However, it took several more years before a conclusive proof of anaerobic oxidation of these compounds was obtained.[14,15] By using ^{14}C-labeled toluene and benzene, $H_2{}^{18}O$, and anaerobic sludge-derived methanogenic consortia, it was shown that the initial transformation step involved oxidation of the aromatic hydrocarbon substrate by oxygen from water (hydroxylation).[14] In toluene, which is an alkylated compound, the oxidation occurs on the methyl group forming an alcohol or on the ring forming a cresol.[15] Further transformation steps overlap with previously established pathways for oxygenated aromatic compounds (alcohols, aldehydes, and acids).[16,17] It is most likely that transition metal complexes in microbial enzymes catalyze oxidative substitution of aromatic hydrocarbons in a similar way as do strong metal oxidants—such as Co(III) fluoroacetate in fluoroacetic acid—that are involved in abiotic reactions of this type, as previously demonstrated.[18,19] Recently, data have become available that show that polynuclear aromatic hydrocarbons are also biodegradable anaerobically, with nitrate as an electron acceptor (soil microcosms),[20,21] as well as under methanogenic conditions (sludge-derived consortia).[22]

In 1983, Schwarzenbach et al. first observed selective removal of toluene and xylenes relative to other components of landfill leachate in the anaerobic zone of a contaminated groundwater aquifer.[23] Their observations were quickly followed by almost identical findings for a different groundwater aquifer by Reinhard et al.[24] These were the first indications that groundwater microorganisms might be capable of transforming homocyclic aromatic hydrocarbons under anaerobic conditions. Furthermore, because no increase in the concentration of reduced alicyclic rings was observed in those aquifers, there was a possibility that the transformation was starting with an oxidation rather than a reduction as typical for oxygenated aromatic compounds. In 1985, Kuhn et al. presented evidence that the three xylene isomers were degraded by denitrifying bacteria in laboratory microcosms filled with subsurface sediments.[25] More detailed studies of toluene and *m*-xylene degradation under denitrifying conditions followed,[26] and the denitrifying pathway for toluene mineralization was soon delineated;[27] this pathway was very close to the methanogenic toluene-degrading pathway previously proposed by Grbic-Galic and Vogel for anaerobic sludge-derived consortia.[15] Parallel with this work, Wilson et al.[25] found that methanogenic aquifer microcosms, derived from a landfill leachate-contaminated groundwater aquifer, degraded benzene, toluene, ethylbenzene, and *o*-xylene; ^{14}C-labeled toluene was mineralized to $^{14}CO_2$.[28] In a subsequent paper, *m*-xylene was added to this list;[29] the microcosms were derived from an aviation gasoline–contaminated aquifer. Other publications followed, presenting benzene degradation by aquifer microorganisms under denitrifying conditions,[30] toluene and *p*-xylene transformation with sulfate possibly acting as an electron acceptor,[31] and the first finding of toluene degradation by groundwater bacteria with Fe(III) as an electron acceptor by Lovley et al. in 1989.[32] The most recent discovery is a pure culture of a denitrifier (*Pseudomonas* sp.), originally derived from a groundwater aquifer, which mineralizes toluene and *m*-xylene.[33]

The information on anaerobic degradation of heterocyclic aromatic hydrocarbons containing nitrogen in their structure, such as indole, quinoline, and isoquinoline, is quite extensive. These hydrocarbons have been demonstrated to degrade under denitrifying,[34] methanogenic,[4,34,35] and sulfate-reducing conditions,[36] and the mechanism for the initial oxydation was shown to be identical to that for homocyclic aromatic hydrocarbons, namely, incorporation of oxygen from water into the aromatic substrate.[37] Furthermore, methanogenic aquifer microorganisms were shown to partially transform these compounds[5] or to completely mineralize them[6,38] in laboratory microcosms containing aquifer material contaminated by creosote. Benzothiophene, a sulfur heterocycle, is also degraded by such microcosms.[39]

In this chapter, we summarize the most recent results of our studies on anaerobic degradation (under methanogenic conditions) of toluene and *o*-xylene and of indene, naphthalene, indole, quinoline, isoquinoline, and benzothiophene by groundwater aquifer–derived microcosms and suspended microbial enrichments.

Materials and Methods

The aquifer material for these studies was derived from a creosote-contaminated groundwater aquifer in Pensacola, FL, at an abandoned wood-preserving plant;[40] it was collected using the continuous-flight auger method.[41] The samples were obtained from a depth of 5–6 m at a site 30 m downgradient from the contamination source. The groundwater at the sampling spot was devoid of dissolved oxygen, was approximately 60–70% saturated with respect to methane, and contained significant amounts of hydrogen sulfide as well as sufficient nitrogen and phosphorus concentrations for microbial activity.[38] Features of the site, aseptic and anaerobic sampling and sample transfer procedures, and the characterization of the aquifer sand have been described elsewhere.[6,38,39]

Toluene (Puresolv TM, scintillation grade) was purchased from Packard Instrument Co. (Downers Grove, IL); *p*-xylene and *m*-xylene (Baker TM grade) were obtained from J. T. Baker Co. (Phillipsburg, NJ). Benzene (99+% pure), ethylbenzene (99+% pure), *o*-xylene (98.2% pure, HPLC grade), naphthalene (99+% pure, scintillation grade), heterocyclic aromatic hydrocarbons (indole, quinoline, isoquinoline, benzothiophene), oxygenated aromatic compounds (phenol, *p*-cresol), and all other chemicals used in these studies were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). ^{14}C-methyl-labeled toluene (purity greater than 98%; specific activity 4.9 mCi/mmol), and ^{14}C-*methyl*-labeled *o*-xylene (purity greater than 98%) were purchased from Sigma Chemical Co. (St. Louis, MO).

The microcosms for the study of homocyclic aromatic hydrocarbon degradation were set up in 250-mL serum bottles sealed with Teflon-coated Mininert valves (Alltech Assoc.). The bottles contained 150 g of aquifer solids, 100 mL of prereduced defined mineral medium with vitamins, and 50 mL of headspace (30% CO_2 and 70% N_2). The medium was modified after Owen et al.[42] by

Table 15.1. Aromatic Substrates Added to Saturated Methanogenic Microcosms with Pensacola, Florida, Aquifer Solids and the Initial Degradation Results (260 Days of Static Incubation at 35°C)

Aromatic Compounds Added and Concentrations (mg/L)	Fate of Aromatic Substrates
Group 1 *Benzene (12)* *Naphthalene* (4)	No degradation of *aromatic hydrocarbons* during 260 days of incubation.
Group 2 *Benzene* (12) *Naphthalene* (4) Phenol (50)	Phenol completely degraded in 80 days of incubation. No degradation of *aromatic hydrocarbons* during 260 days of incubation.
Group 3 *Toluene* (4) *Ethylbenzene* (4) o-*Xylene* (4) p-*Xylene* (4)	*Toluene* completely degraded in 120 days of incubation. *o-Xylene* completely degraded in 255 days of incubation. No degradation of *ethylbenzene* or p-*xylene* during 260 days of incubation.
Group 4 *Toluene* (4) *Ethylbenzene* (4) o-*Xylene* (4) p-Xylene *(4)* *p*-Cresol (5)	*p*-Cresol completely degraded in 80 days of incubation. *Toluene* completely degraded in 100 days of incubation. *o-Xylene* completely degraded in 200 days of incubation. *o-Xylene* degradation starts after the first spike of *toluene* has been completely degraded; the two processes continue in parallel thereafter. No degradation of *ethylbenzene* or p-*xylene* during 260 days of incubation.

Note: Aromatic hydrocarbons are italicized; additional aromatic substrates are not.

reducing concentrations of all inorganic nutrients approximately one order of magnitude; adding zinc, selenium, and aluminum; and using amorphous ferrous sulfide as a reducing agent instead of a combination of sodium sulfide and ferrous chloride. The serum bottles containing the medium were autoclaved prior to microcosm preparation; vitamin solution and the buffering sodium bicarbonate solution were added after autoclaving and were sterilized by filtration through 0.2-μm pore-size microbiological filters. The microcosms were prepared and then incubated at 35°C in an anaerobic glovebox. Eight different combinations of aromatic hydrocarbons, with or without oxygenated aromatic compounds (phenol, *p*-cresol) as accessory substrates, were added as carbon and energy sources for microorganisms. Four groups of microcosms received mixtures of aromatic hydrocarbons (two to four components), with or without oxygenated aromatic compounds, in which each component was present at a concentration of at least 40 mg/L; these microcosms showed no microbial activity toward aromatic hydrocarbons. The other four groups received one order of magnitude lower concentrations of substrates; these four combinations are outlined in Table 15.1. No electron acceptors (except for CO_2) were added to the microcosms, thus creating conditions which should be conducive to fermentation and methanogenesis (after all the natural electron acceptors from the aquifer solids have been depleted). For each combination of the substrates, duplicate active microcosms and one autoclaved biological control (containing all the microcosm constituents) were established and monitored. The degradation of aromatic hydrocarbons, over time, was measured by sampling headspace (for aromatic hydrocarbon analysis) and liquid phase (for polar aromatics analysis) and analyzing the samples of Hewlett-Packard Series II Model 5890A gas chromatograph (Hewlett-Packard, Avondale, PA) with an HNU Model PI 52-02A photoionization detector (HNU Systems, Inc., Newton, MA) and a 30-m × 0.53-mm DB-624 megabore fused silica capillary column with 3-μm film thickness (Durabond, J and W, Inc., Rancho Cordova, CA).

Suspended mixed cultures degrading toluene and *o*-xylene were enriched from the active microcosms by transferring 20 mL of the liquid and approximately 10 g of the solid portion of the microcosms into 180-mL of defined anaerobic mineral media with toluene, *o*-xylene, or a combination of these substrates (4 mg/L each). After the degradation of the aromatic substrates had gone to completion, the cultures were refed 5 mg/L of each of the substrates, and upon completion of degradation of this secondary spike were transferred again (30 mL, after vigorous shaking) into 170 mL of fresh media with 5 mg/L of the respective substrates (single or in binary mixture). ^{14}C-labeled toluene and xylene were recently spiked to the suspended cultures in order to determine the mass balance of toluene and xylene degradation; the initial concentration of the label was 1000 dpm/mL for each compound. The ^{14}C activities in the headspace and the culture fluid were measured using Tri-Carb liquid scintillation system (No. 4530; Packard Instrument Co.), according to the procedure described by Grbic-Galic and Vogel.[15]

Microcosms for studies of methanogenic transformation of polynuclear, heterocyclic (indole, quinoline, isoquinoline, and benzothiophene), and homocyclic aromatic hydrocarbons (indene, naphthalene) were prepared in 500-mL serum bottles with approximately 400 g of aquifer solids and 250 mL of prereduced defined mineral medium as described by Godsy and Grbic-Galic.[39] Single hydrocarbons were added to the microcosms as sole organic carbon and energy sources in the concentration of 10–40 mg/L. Sorption of the examined compounds to aquifer solids had been found to be negligible.[6] Duplicate microcosms, autoclaved biological controls, and live controls unamended with aromatic hydrocarbons were monitored in parallel using daily sampling and analysis procedures. Gas chromatography (GC), mass spectrometry (GC-MS), and high-performance liquid chromatography (HPLC) techniques, used to monitor the disappearance of substrates, formation and transformation of intermediates, and production of final products, are described elsewhere.[6,38,39] In addition to the small microcosms, a large anaerobic microcosm containing approximately 4 kg of aquifer material and 2.5 L of contaminated groundwater from the site was set up in a 4-L hermetically sealed glass bottle equipped with gas- and liquid-sampling ports and a U-tube manometer.[6] The water in this microcosm was amended with amorphous ferrous sulfide as a reducing agent. The microcosm was used to follow the fate of some of the hydrocarbons listed above when present in a mixture (a simulation of the situation in the field). Gas production and composition, substrate transformation, and intermediate formation and degradation were monitored at 7-day intervals by using GC, HPLC, and GC-MS.[6] All the microcosms were incubated in an anaerobic chamber at 22°C.

Results and Discussion

Methanogenic Degradation of Homocyclic Aromatic Hydrocarbons[43]

As indicated in Table 15.1, methanogenic microcosms derived from the Pensacola aquifer underwent a long acclimation period before the onset of aromatic hydrocarbon degradation. Once the activity was enriched, 4–5 mg/L of toluene or *o*-xylene were degraded in approximately 2 weeks; initially, however, about 100–120 days were required for complete degradation of toluene, and 200–250 days for *o*-xylene. The initial lag period may be due to small populations originally present on the aquifer solids, especially when the microcosm size is so small, and to slow growth rates typical of anaerobic microbial communities. It may also be due to the absence of the required enzymatic activity, to the necessity of induction or a genetic event that would trigger the right type of catalysis, or to various other reasons.[44] We are currently experimenting with the fresh, unacclimated aquifer material from the same source in an attempt to find out whether the addition of higher concentrations of toluene (10 mg/L) or high concentrations of accessory carbon sources (100 mg/L *p*-cresol, 150 mg/L acetate) would accelerate the buildup of the aromatic-degrading community and decrease the initial lag.

The results in Table 15.1 suggest that the addition of *p*-cresol accelerates the onset of degradation of toluene and *o*-xylene. *p*-Cresol had been shown previously to be an early intermediate in degradation of toluene by anaerobic sludge-derived methanogenic consortia;[14,15] it is conceivable that this compound plays the same role in groundwater-derived methanogenic microcosms (Table 15.2). Our current work, which includes enrichment and isolation of pure cultures from toluene- and *o*-xylene-degrading microcosms, will hopefully elucidate the *p*-cresol effect.

Toluene and *o*-xylene were the only aromatic hydrocarbons degraded by these consortia; benzene, ethylbenzene, other xylene isomers, and naphthalene were not. The available literature on anaerobic degradation of aromatic hydrocarbons by aquifer microorganisms generally indicates that alkylated aromatic hydrocarbons are more easily degraded than the unsubstituted ones. There are also indications that the activity is site specific and that the compounds that are degraded in one aquifer will not necessarily be degraded under similar conditions in another aquifer. The microbial activity of this type is not ubiquitous. If the process application (*in situ* treatment) is considered, the first step with each specific site should be laboratory investigations of the indige-

Table 15.2. Homocyclic and Heterocyclic Aromatic Hydrocarbons Examined in This Study and Some of the Products of Their Methanogenic Degradation

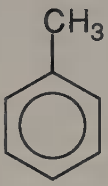
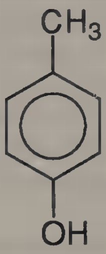
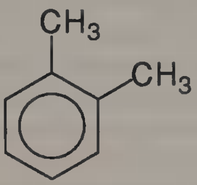
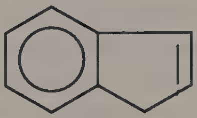
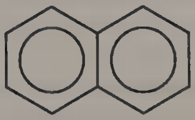
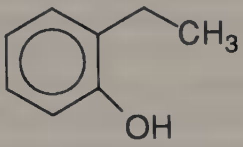
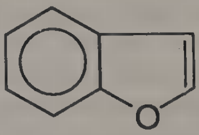
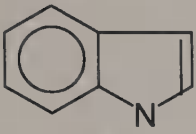
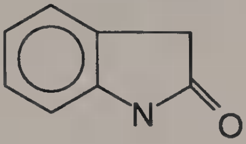
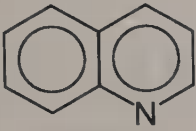
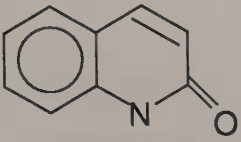
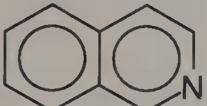
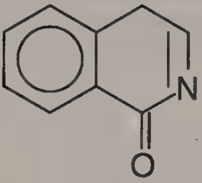
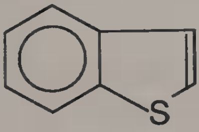
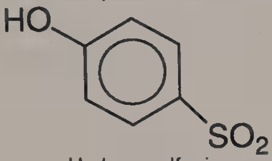
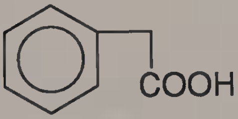
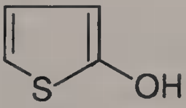
Aromatic Hydrocarbons	Earliest Oxidation Intermediates Detected	Final Products
 Toluene	 *p*-Cresol[a]	CO_2, CH_4
 *o*-Xylene		CO_2, CH_4
 Indene		CO_2, CH_4
 Naphthalene	 2-Ethylphenol[a]  Benzofuran[a]	CO_2, CH_4

Table 15.2, continued

Aromatic Hydrocarbons	Earliest Oxidation Intermediates Detected	Final Products
 Indole	 Oxindole	CO_2, CH_4
 Quinoline	 Quinolinone	CO_2, CH_4
 Isoquinoline	 Isoquinolinone	CO_2, CH_4
 Benzothiophene	 *p*-Hydroxysulfonic acid	CO_2, CH_4
	 Phenylacetic acid	CO_2, CH_4
	 Thiophene-2-ol	

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[a] Intermediates not found in this study but indirectly implied (*p*-cresol from toluene) and detected in sludge-derived methanogenic consortia metabolizing the same aromatic hydrocarbon substrates.[14,15,22]

nous microorganisms and their capabilities under simulated aquifer conditions.

It is interesting to note that the examined microbial communities showed high specificity for only one (*o*-) of the three xylene isomers. Wilson et al., in contrast, observed degradation of both *o*-and *m*-xylenes by methanogenic microcosms derived from another contaminated groundwater aquifer.[29] The composition of microflora from these two sites is obviously different; groundwater chemistry and availability of specific nutrients might be among the numerous possible causes that could determine the makeup of the microbial communities.

Suspended, stable, mixed methanogenic cultures (consortia) were enriched from the active microcosms and tested for their capability to degrade toluene and *o*-xylene as sole organic carbon and energy sources. The results of these preliminary experiments are shown in Figures 15.1 and 15.2. One of the cultures was fed only toluene, another only *o*-xylene, and the third a mixture of these two compounds. In the microcosms, toluene was degraded first and *o*-xylene (a more complex substrate) second; in the acclimated suspended cultures the degradation of both compounds occurred simultaneously. Furthermore, the binary mixture does not seem to affect the degradation of either of the two compounds—the rates of removal of toluene and *o*-xylene as sole substrates are the same as the rates for the respective compounds in the mixture. Methane production occurs in all of the suspended cultures, with the amount of methane produced approaching very closely the stoichiometrically expected values. Isolation of pure cultures from the suspended consortia, which is under way, will help understand the community structure and answer

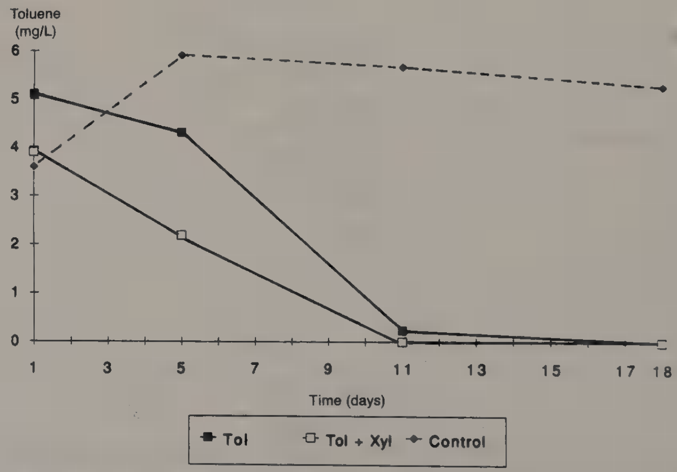

Figure 15.1. Toluene degradation by suspended mixed methanogenic cultures containing toluene or a mixture of toluene and xylene as carbon and energy sources. The chemical control contains both toluene and xylene. *Tol* = toluene; *Tol + Xyl* = toluene plus xylene.

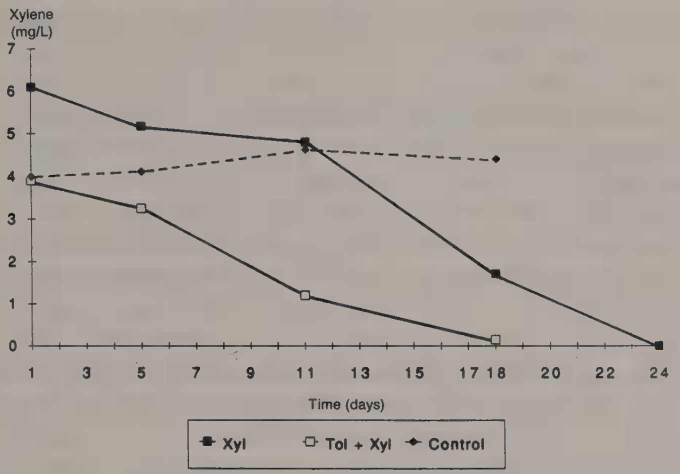

Figure 15.2. Xylene degradation by suspended mixed methanogenic cultures containing xylene or a mixture of toluene and xylene as carbon and energy sources. The chemical control contains both toluene and xylene. *Xyl* = xylene; *Tol + Xyl* = toluene plus xylene.

the question of whether the toluene and *o*-xylene degradation are catalyzed by the same group of microorganisms or by two different groups working simultaneously. We have also just started the use of ^{14}C-labeled toluene and *o*-xylene; this will enable us to perform accurate mass balances.

Methanogenic Degradation of Homocyclic and Heterocyclic Polynuclear Aromatic Hydrocarbons[6,38,39,45]

Transformation of indole (initial concentration 40 mg/L) in small microcosms fed this heterocycle as the sole organic carbon and energy source, started without a lag, and was completed in 25 days. The formation of oxindole, the earliest intermediate of indole oxidation (Table 15.2), started on day 6 and by day 16 reached the expected stoichiometric maximum, indicating complete conversion of indole. Oxindole persisted for a month, but afterwards was completely mineralized to CO_2 and CH_4 (day 53). Quinoline (initial concentration 20 mg/L) as sole substrate was oxidized after a lag period of 20 days; the degradation was completed in 55 days after the beginning of the incubation period. Isoquinoline was oxidized completely in 60 days; this period included 35 days of the initial lag. Quinolinone and isoquinolinone, the early transformation intermediates of quinoline and isoquinoline, respectively (see Table 15.2), were formed in stoichiometric amounts from their parent compounds; they persisted until quinoline and isoquinoline were completely removed and then were degraded, ultimately to carbon dioxide and methane. Additional transformation intermediates detected by HPLC and GC-MS in quinoline-degrading microcosms included 2,3-dimethylpyridine, aniline, benzoic acid, phenol, 1,9-nonanediol, nonanoic acid, and octanoic acid.[38,45] These intermediates indicate that the degradation of quinoline is initiated through introduction of an oxygen function (hydroxy group), most likely from water, and that subsequent degradation steps include side chain transformations and ring cleavage reactions typical of anaerobic degradation of oxygenated aromatic compounds.[16,17] Compounds like 2,3-dimethylpyridine suggest that not only the heterocyclic but also the homocyclic ring of quinoline may be the site of the initial oxidative attack. Similar results were obtained with benzothiophene, a sulfur heterocycle (10 mg/L initial concentration), whose degradation was started after a 12-day lag and was completed by day 24. We were not able to detect the earliest intermediate of benzothiophene transformation, but a whole series of oxidized mononuclear aromatic intermediates, both homocyclic and heterocyclic, were identified in the culture fluid: *p*-hydroxysulfonic acid, thiophene-2-ol, phenylacetic acid (see Table 15.2), 2-hydroxyphenylacetic acid, 2-oxophenylacetic acid, benzyl alcohol, benzoic acid, and phenol.[39,45] In addition to the aromatic intermediates, alicyclic compounds (cyclohexyl alcohol, cyclohexanecarbocylic acid) and aliphatics (2-methyl-2-hexanol, 3-hexenol, 2-methyl-1,2-propanediol, thiopropionic acid, hexanoic acid, 2-hexenoic acid) were also detected. All these intermediates were transient and completely disappeared by the end of the fourth week of incubation, when the substrate was ultimately converted to stoichiometric amounts of CO_2 and CH_4.

It is interesting to note that the early oxidation intermediates of nitrogen heterocycles (oxindole, quinolinone, isoquinolinone) persisted for a long time in the laboratory microcosms (and in the field, see the following), whereas the early oxidation intermediate of benzothiophene was further transformed so fast that we could not even detect it in the culture fluid. These results suggest that the nitrogen heterocycles are probably initially transformed by microorganisms different from those that transform sulfur heterocycles. The results also suggest that the populations that are responsible for degradation of oxidized nitrogen heterocycles are possibly present in very low numbers in the aquifer material and need to build up before the transformation of these compounds can be observed experimentally. This phenomenon is currently under investigation.

Indene and naphthalene, fed as individual substrates to the smaller microcosms, were completely degraded in from 1 (naphthalene) to 2 months (indene) with stoichiometric production of carbon dioxide and methane.[22] No intermediates were detected. It is interesting to note that microcosms of an even smaller size (150 g of aquifer solids, 100 mL of medium) used in a separate experiment, as well as the microcosms described previously in the section on toluene and *o*-xylene degradation (also containing only 150 g of aquifer solids and 100 mL of medium), did not degrade naphthalene in 10 months of incubation. The same was observed with benzothiophene. These results emphasize the impact of the small microbial numbers present in the subsurface and of the heterogeneity in microbial distribution within an aquifer. Smaller microcosms may easily be devoid of microorganisms that are crucial for the success of certain transformation processes.

The results from the largest microcosm tested (4 kg of aquifer solids, 2.5 L of contaminated groundwater) indicate that quinoline and isoquinoline are completely removed during the first 40 days of incubation; quinolinone and isoquinolinone take 150 and 180 days, respectively, but are also completely degraded to CO_2 and CH_4. Quinoline and isoquinoline degradation occurs simultaneously with the degradation of some of the other components in the contaminated groundwater—benzoic acid and volatile fatty acids. Quinoline and isoquinoline degradation precedes the degradation of phenol and cresols (sequential degradation in a complex mixture).[6] Acetic acid seems to be an intermediate in the methanogenic degradation of all of these compounds; it peaks shortly after benzoic acid and volatile fatty acids are eliminated and then peaks again concurrently with the removal of quinolinone, isoquinolinone, and cresols.[6] These findings can be conveniently compared with the observations from the field (Figure 15.3) because the groundwater velocity at the depth of 5–6 m is about 1 m/day and the distance traveled downgradient from the contamination source equals approximately the residence time in the large microcosm. The microcosm results simulate very closely the results of field measurements: quinoline and isoquinoline decrease disproportionately downgradient when compared to the conservative tracer (3,5-dimethylphenol)[38] and are completely removed within the first 50 m downgradient from the source, whereas their early oxidation intermediates, quinolinone and isoquinolinone, persist for about 200 m before they are eliminated. Furthermore, acetic acid, suggested to be an important intermediate in the laboratory methanogenic microcosms, temporarily increases also during the downgradient movement in the aquifer as creosote constituents are being degraded (not shown in the figure).[6] Figure 15.4 shows the behavior of homocyclic polynuclear aromatic hydrocarbons (PAH)—indene, naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, and acenaphthene. Although the laboratory experiments indicated successful degradation of both indene and naphthalene to carbon dioxide and methane, field observations suggested efficient selective transformation of indene only, while the other PAH compounds remained persistent within the 200 m of the test zone. The difference between the laboratory and field results for PAH compounds (except for indene) does not necessarily mean that these compounds are not biodegradable in the field, but probably that they are not degraded during downgradient travel in this particular section of the aquifer.

Conclusions

The indigenous methanogenic communities from the Pensacola, Florida, groundwater aquifer (creosote contaminated) are capable of degrading two simple monoaromatic hydrocarbons—toluene and *o*-xylene—in laboratory microcosms and suspended cultures. Once the microbial cultures are adapted

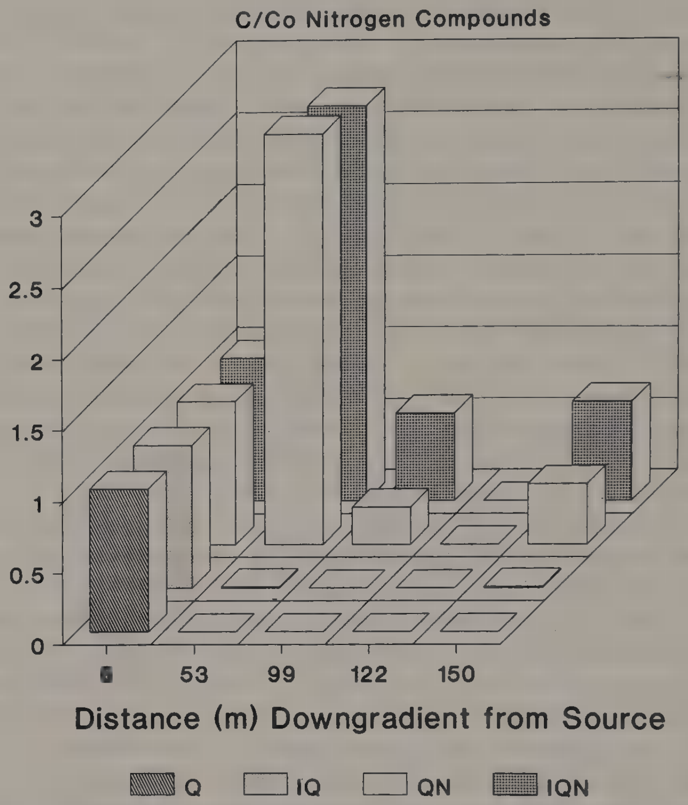

Figure 15.3. Concentration of nitrogen heterocyclic compounds relative to the concentration of the conservative tracer (3,5-dimethylphenol), downgradient from the contamination source. *Q* = quinoline; *IQ* = isoquinoline; *QN* = quinolinone; *IQN* = isoquinolinone.

to the aromatic hydrocarbon substrates, the degradation occurs quickly (complete conversion to CO_2 and CH_4 in less than 2 weeks). Other compounds degraded include some constituents of the water-soluble fraction of creosote (indene, naphthalene, indole, quinoline, isoquinoline, and benzothiophene); the degradation of some of these compounds has been observed and measured also in the field. The fact that the number of compounds degraded increases with the microcosm size, stresses the importance of sufficiently abundant and diverse biomass in biodegradation of these contaminants under methanogenic conditions. This is important to keep in mind when designing possible *in situ* treatment; nutrient addition to encourage microbial growth might prove helpful.

According to the results of laboratory experiments with some of the compounds (indole, quinoline, isoquinoline, and benzothiophene), the initial transformation step is oxidative, and the oxygen for this reaction is probably

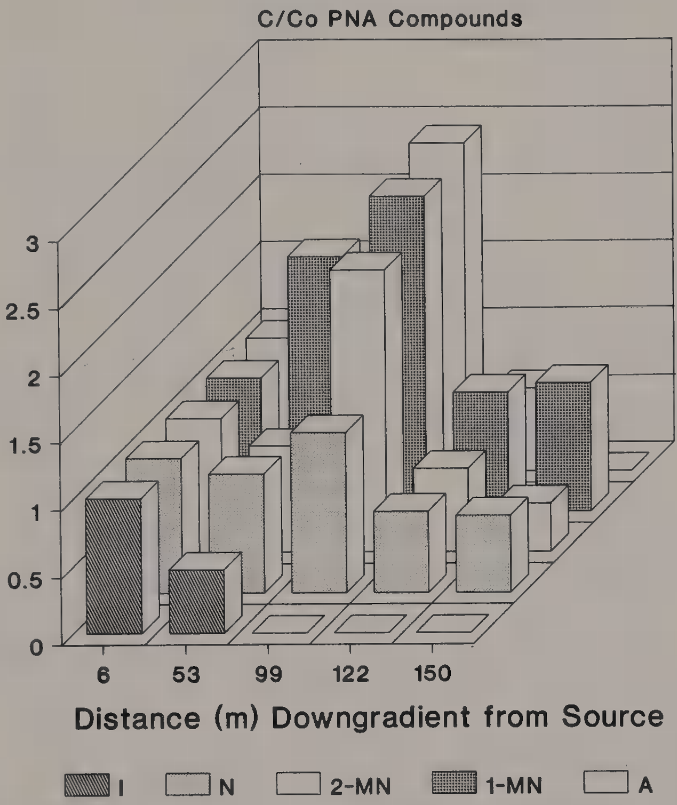

Figure 15.4. Concentration of homocyclic PAH relative to the concentration of the conservative tracer, downgradient from the contamination source. *I* = indene; *N* = naphthalene; *2MN* = 2-methylnaphthalene; *1MN* = 1-methylnaphthalene; *A* = acenaphthene.

derived from water. A conclusive proof of this possibility was provided by Pereira et al. using $H_2{}^{18}O$ in sludge microcosms transforming quinoline.[37] After the initial oxidation, the transformation pathways overlap with the pathways previously suggested for oxygenated aromatic compounds. In general, the overall pathways for polynuclear heterocyclic compound transformation seem to be similar to the degradation routes for mononuclear homocyclic aromatic hydrocarbons under methanogenic conditions.[14,15]

AEROBIC MICROBIAL TRANSFORMATION OF TRICHLOROETHYLENE

Background

In 1985, Wilson and Wilson suggested that methanotrophic communities from soil might be responsible for aerobic degradation of trichloroethylene (TCE).[12] This finding resulted in an explosion of research on the topic, and soon results were published that indicated that mixed cultures consisting of methanotrophs and heterotrophs—which were quite common in soil, sediments, and the subsurface—could completely mineralize this compound to carbon dioxide, chloride, and water.[46-50] The first step of TCE transformation by methanotrophs is most likely an epoxidation to TCE-epoxide, catalyzed by methane monooxygenase (an enzyme with a very broad substrate specificity), as first proposed by Henry and Grbic-Galic.[47] The epoxide is extremely unstable in aqueous solution and breaks down into various products that can be degraded by heterotrophs. Several researchers have succeeded in isolating pure cultures of methanotrophs that transform TCE,[51,52,53] or in detecting and analyzing the activity in known methanotrophic strains.[54,55] In addition to methanotrophs, other bacteria are capable of fortuitous transformation of this compound, such as ethylene oxidizers,[52,65] propane oxidizers[56] and pseudomonads that grow on aromatic compounds.[57-60]

The investigations at Stanford University of TCE transformation by methanotrophs started in the form of parallel efforts in the field and in the laboratory. The field experiment (Moffett Field Naval Air Station, Mountain View, CA) concentrated on stimulation of growth of the indigenous methanotrophic communities by injecting methane and oxygen into the aquifer; the resulting activity toward TCE and other halogenated aliphatics was then observed.[61] In the laboratory, detailed studies were undertaken in microcosm systems and with suspended mixed and pure methanotrophic cultures in order to learn about the relevant microorganisms and about the prerequisites and optimal conditions for the TCE transformation process. Some of these laboratory studies are described in the following.

Materials and Methods

Microcosms Containing Aquifer Solids[62-64]

The aquifer solids were obtained from a pristine part of the Moffett Naval Air Station site from a depth of 5–7 m, using a hollow-stem auger drilling rig.[63] The solids consisted of a fine to coarse sand, gravel, and minor clay. The inner portion of the core was aseptically removed with a sterilized metal pipe, and the <2-mm size fraction used to pack six glass columns (40 × 2 cm). The columns were saturated with air-stripped and filter-sterilized groundwater from the site; the same water was used for all the subsequent exchanges.[62] Each of the columns received 50 μg/L of ^{14}C-labeled TCE. Four of the columns received dissolved methane; two did not (controls). Two of the methane-fed columns (5 mg/L dissolved methane) and one of the two control columns were amended with 22 mg/L of dissolved oxygen (the water had been saturated with O_2 by bubbling pure oxygen through it for an hour before use), while the other two methane-fed columns (saturated methane solution) and the remaining control received hydrogen peroxide (85 mg/L) instead of oxygen. The 22 mg/L of dissolved oxygen in the O_2-amended columns were sufficient to maintain aerobic conditions even after all the methane was depleted. All the columns (incubated at 22°C) were operated as sequential batch reactors at the exchange intervals of 3 days to 2 weeks. The maintenance of the columns and exchanges and the establishment of a breakthrough curve using bromide, dissolved oxygen, and dissolved methane as tracers have been described elsewhere.[62,63] The effluent was analyzed for methane concentration (by GC), dissolved oxygen (by dissolved oxygen probe), ^{14}C-labeled TCE and other ^{14}C-labeled compounds (by GC and scintillation counting), and hydrogen peroxide (by peroxide test strips, EM Science, and titration with ceric sulfate).[62-64]

Additional experiments were performed in 35-mL batch microcosms that were periodically sacrificed for measurements.[64] The microcosms were completely filled, without headspace, and contained 1.5 g of well-mixed aquifer solids from the columns, 2 mL column effluent, and a mineral medium with 4.5 mg/L of dissolved methane, 24 mg/L of dissolved oxygen, and 50 μg/L of TCE. At each measuring interval, triplicate microcosms were sacrificed. Each of them was analyzed for methane, dissolved oxygen, ^{14}C-labeled TCE, and $^{14}CO_2$. Batch microcosms were used to study the interaction between the concentration of methane present and TCE degradation and the influence of formate and methanol addition on the TCE transformation under no-growth conditions.

Suspended Mixed and Pure Cultures[65]

Mixed cultures consisting of methanotrophic and heterotrophic bacteria were enriched from aquifer solids or groundwater from the Moffett Field site. Continuously stirred reactors containing 1 L of defined mineral medium[65] under a continuous flow of 25% methane in air were inoculated with approximately 1 g of aquifer solids or 20 mL of groundwater. The reactors were then incubated at room temperature. Stable enrichments were obtained by five to ten sequential transfers to new reactors and were confirmed by microscopic and macroscopic observations (consistent micromorphology and macromorphology). A pure methanotrophic culture was obtained from one of the stable enrichments by streaking dilutions of the mixed culture on agarose plates with defined mineral medium and incubating the plates in an atmosphere of approximately 25% methane in air. Culture purity was proven by growth in defined mineral medium with methane as the sole organic carbon and energy source, by the absence of growth on multicarbon substrates, and by scanning and transmission electron microscopy.[52,65]

Three stable suspended mixed cultures and the pure culture were tested for their capabilities to transform TCE by transferring actively growing cultures from the reactors (in which they were incubated under a continuous stream of approximately 35% methane in air) to shake flasks. The bottles of degradation studies were incubated upside-down on a shaker at 21°C. In addition to the defined mineral medium cited above, Whittenbury mineral medium[66] was used for some of the experiments. The effects of CH_4, O_2, and TCE concentrations, reducing power availability, and mineral medium formulation on TCE transformation were studied. The techniques used in these experiments are extensively described elsewhere,[52,53,65,67–71] and include cell biomass determination on a dry-weight basis; staining with sudan black B for lipid storage granules;[72] radiotracer experiments with ^{14}C-labeled TCE; sampling of culture headspace for unlabeled TCE and analysis on a Tracor GC Model MT-220 DPFFN (Tracor, Austin, TX), equipped with an electron capture detector (method developed by Criddle[10]); gas partitioning for analysis of CO_2, O_2 and CH_4 in the headspace; evaluation of acid intermediates on a Dionex ion chromatograph Series 4000i (Dionex Corporation, Sunnyvale, CA); and detection of carbon monoxide as a TCE transformation product on a Trace Analytical RGD2 reduction gas detector (Trace Analytical, Menlo Park, CA).

The maximum TCE transformation rate coefficient k (d^{-1}), the half-saturation constant K_s (mg/L), and second-order rate coefficient k/K_s (L/mg-day) were determined using Monod kinetics for substrate degradation, $-dS/dt = kXS/(K_S + S)$, where S equals TCE concentration (mg/L); X, biomass concentration (mg/L); and K_s, half-saturation coefficient for TCE (mg/L). Except where noted, no methane was added, and it was assumed that the biomass concentration, X, was constant during the duration of the experiments. The correlation coefficient for the fit of the data to the model was greater than 0.95.[65] The mass of TCE was converted to aqueous concentration using a dimensionless Henry's constant of 0.33 at 21°C.

Results and Discussion

Microcosms Containing Aquifer Solids[62,64]

After 3 months of feeding methane and oxygen (in which period an active methanotrophic community was successfully established), the columns were amended with TCE. The added TCE was strongly sorbed to the aquifer solids so that only half of ^{14}C-labeled TCE could be recovered in the effluent after the treatment period.[62] After nearly 1 year of operation, the stabilized communities in the columns degraded 15 to 20% of TCE to $^{14}CO_2$; no activity toward TCE was observed in the control column, which was fed no methane. These results agree very well with the results from the field,[61] where 20% removal of TCE was the maximum value observed; sorption seems to be the major limiting factor. No transformation products other than CO_2 were observed in the column effluent.

The addition of hydrogen peroxide as an oxygen source decreased the methane consumption. Whereas oxygen-amended columns consumed 98% of the added methane in the 3-day exchange period, the hydrogen peroxide–amended ones consumed only 87%. However, more oxygen was utilized by bacteria in the H_2O_2-amended columns than in the oxygen-amended columns, indicating that hydrogen peroxide had a negative impact on the methanotrophs, possibly enabling the heterotrophs to outcompete the methanotrophs for the available oxygen. The high oxygen concentrations generated by H_2O_2, rather than the H_2O_2 itself, could have been the agent that caused the inhibition of the methanotrophic activity. In the experiments with suspended microbial cultures, high oxygen inhibited TCE transformation (see the following section).[65] No TCE-degradation experiments were performed in the H_2O_2-amended columns.

Batch microcosm results indicated temporal separation of methane utilization and TCE transformation. This suggests a possible competition between methane and TCE for the active sites on the same enzyme (see the following section) and the continuation of the enzyme activity in the absence of the growth substrate, probably due to utilization of reducing power from the internal bacterial storages, as suggested by Henry et al.[52] The addition of formate (an energy source for methanotrophs) and, to a lesser degree, methanol (Figure 15.5) increased the amount of TCE converted to CO_2 in the absence of methane, emphasizing the importance of an accessory energy (reducing power) source for TCE degradation by methanotrophic communities.

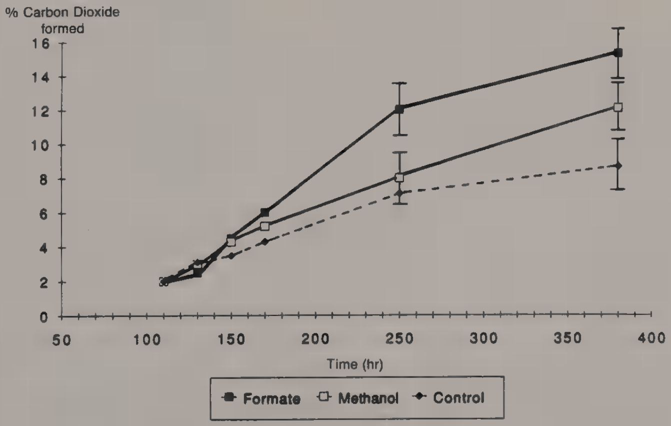

Figure 15.5. The effect of formate and methanol addition on TCE transformation by batch microcosms in the absence of methane. Each data point represents the mean of measurements on triplicate microcosms. Bars represent one standard deviation. Modified after Lanzarone et al.[763]

Suspended Mixed and Pure Cultures[47,52,53,65,67–71]

Three micromorphologically and macromorphologically different methane-grown mixed cultures were enriched from the Moffett Field groundwater aquifer: culture MM1 from aquifer solids; culture MM3 from groundwater; and culture MM2 from the effluent of a TCE-degrading, methane-enriched column packed with the aquifer solids.[63] All of them consisted of various gram-negative bacteria; MM2 also contained a fungus (yeast). MM1 and MM3 contained only type II methanotrophs; MM2 contained both types I and II. The methanotrophs in mixed cultures MM1 and MM3 had lipid storage granules; methanotrophs in the mixed culture MM2 did not.[52,53,65,69] A pure culture of a type I methanotroph was isolated from MM2 and characterized as a *Methylomonas* sp. strain MM2.[53] The pure culture grew well on methane and methanol but did not grow on multicarbon substrates; it lacked lipid storage granules.

MM1, MM2, MM3, and *Methylomonas* sp. strain MM2 all transformed TCE in the presence of methane as well as in its absence (no-growth conditions). Rates varied from 2.3 L/mg-day to 0.003 L/mg-day (second-order rate coefficient k/K_s), depending on culture characteristics, growth, and incubation conditions. Coefficient K_s (mg/L), which can be interpreted to represent the affinity of the enzyme for TCE, varied with culture and growth conditions, ranging from 0.2 to 1.3 mg/L.[65,70] When sufficient reducing power was available, 100% of the TCE was transformed. Acetylene inhibited TCE oxidation and methane oxidation. Methane competitively inhibited TCE oxidation. The main product in the mixed cultures was CO_2 (mineralization); a significant portion of carbon from ^{14}C-labeled TCE was associated with the biomass, and a smaller fraction (5–10%) remained in nonvolatile aqueous products. In the pure culture, 70–80% of the TCE carbon remained in nonvolatile aqueous intermediates, 10–20% was associated with the biomass, and the rest was converted to CO_2.[65] Three unidentified acids were present in the aqueous intermediate fraction. Standard coelution on the ion chromatograph confirmed that these acids were not monochloroacetate, dichloroacetate, trichloroacetate, or glyoxylate. Both formate and carbon monoxide were found to be transformation intermediates, and both were oxidized by the pure culture.[71]

Mixed cultures MM1 and MM2 and the pure culture *Methylomonas* sp. strain MM2 were tested for their capability to transform TCE with no, low, and high concentrations of methane present (0, 0.45, and 4.5 mg/L initial aqueous concentration). The high concentration of methane slowed down TCE transformation, as would be expected because of the phenomenon of competitive inhibition. However, MM2 and the pure culture exhibited significantly slower rates of TCE transformation in the absence of methane. This corresponds to their lack of lipid storage granules.[69] Lipid inclusions–containing MM1, in contrast, transformed TCE equally well, both in the presence and absence of methane. The methane monooxygenase system, which is responsible for the TCE oxidation, requires a source of reducing power to reduce the residual atom from the oxygen molecule to water.[73] Lipid storage granules (an internal source of reducing power) should facilitate the transformation process in the absence of the growth substrate.[65,69] In another experiment, the cultures that contained lipid inclusions (MM1 and MM3) remained active toward TCE for a longer time under starvation conditions than did the culture that lacked lipid granules (MM2).[69] The addition of formate (an auxiliary reducing power source) to methane-starved *Methylomonas* MM2 cells increased the TCE transformation rate and enabled the microorganism to sustain the TCE transformation longer than in the absence of formate.[69]

Oxygen is required by methanotrophs both for respiration and for the oxidative function of the methane monooxygenase. Many methanotrophs, however, have been described as microaerophilic and grow best at oxygen tensions below atmospheric. High oxygen concentrations at 50% of headspace were inhibitory, reducing TCE transformation by approximately 20% in all the cultures tested. Oxygen at 35% was not inhibitory. When cultures were incubated under near-anoxic conditions ($<1\%$ O_2), very little methane or TCE was oxidized. When oxygen was depleted, TCE transformation stopped.[65] High concentrations of TCE were toxic to the cultures. At TCE concentrations up to 10 mg/L, all the methane present was utilized, and all the TCE transformed by mixed cultures. At 44 mg/L of TCE, no methane was utilized and only 20% of the TCE was transformed.[65] There were indications that the TCE oxidation itself was toxic to the pure culture. The *Methylomonas* sp. strain MM2 cells that oxidized TCE maintained at a concentration equal to or not greater than 6 mg/L were significantly impaired in their capability to oxidize methane in a subsequent experiment.[67]

Carbon monoxide, a TCE transformation product which was oxidized by *Methylomonas* sp. strain MM2, had a significant inhibitory effect on TCE oxidation. This was due, at least in part, to the competitive inhibition exerted by carbon monoxide.[71]

The mineral medium for microbial growth influenced the TCE transformation rate significantly. Growth in Whittenbury mineral medium,[66] which contained EDTA (a metal chelator) and FeEDTA, resulted in a three-fold (*Methylomonas* sp. strain MM2) to 15-fold (MM1) increase in the rate of TCE transformation.[53,65] Omission of EDTA from the Whittenbury mineral medium resulted in an order-of-magnitude decrease in rates of TCE transformation. EDTA may be affecting the availability of a required metal, or sequestering an inhibitory one.

Conclusions

Different mixed methanotrophic/heterotrophic cultures transforming TCE were isolated from the Moffett Field aquifer when different selection procedures were used. This indicates a great heterogeneity of microbial communities in the subsurface. The site was not initially contaminated with TCE; the capabilities of the indigenous methanotrophs to tackle this compound are due not to adaptation but to the broad substrate specificities of their methane monooxygenases. Methanotrophs are crucial both as members of the food chain and as catalysts in the degradation of TCE. They support heterotrophs with their metabolic by-products, and by oxidizing TCE they promote the formation of TCE transformation intermediates that can be mineralized by the heterotrophs.

In process applications, methanotrophs may be exposed to methane depletion either accidentally or intentionally as a part of the process design. Formate addition would provide a source of reducing power during such periods of starvation and might serve to both increase TCE transformation rates and prolong TCE transformation in the absence of methane. In the field experiment, addition of formate resulted in increased removal of TCE during periods of methane depletion.[61] Methanotrophs with storage polymers such as lipid inclusions contain an endogenous reserve of reducing power and may survive starvation and transform TCE during starvation for longer periods than those that lack storage polymers. Such methanotrophs may be well suited for process applications in which methane depletion occurs. It may be desirable to seed reactors with lipid inclusions–containing methanotrophs and to promote the growth of such methanotrophs in field applications.

The aquifer methanotrophs used in these studies were sensitive to hydrogen peroxide exposure and to high concentrations of oxygen. The potential for inhibition should be taken into consideration if H_2O_2 or pure oxygen is used in methanotrophic treatment applications. The pronounced influence of the growth medium formulation on TCE transformation rates indicates that the groundwater chemistry will be very important in successful *in situ* treatment; this phenomenon, as well as the phenomena of TCE oxidation toxicity and inhibition of TCE transformation by the degradation product carbon monoxide, should be considered in the design of process applications.

ACKNOWLEDGMENTS

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CHAPTER 16

Biodegradation of Organic Contaminants in Sediments: Overview and Examples with Polycyclic Aromatic Hydrocarbons

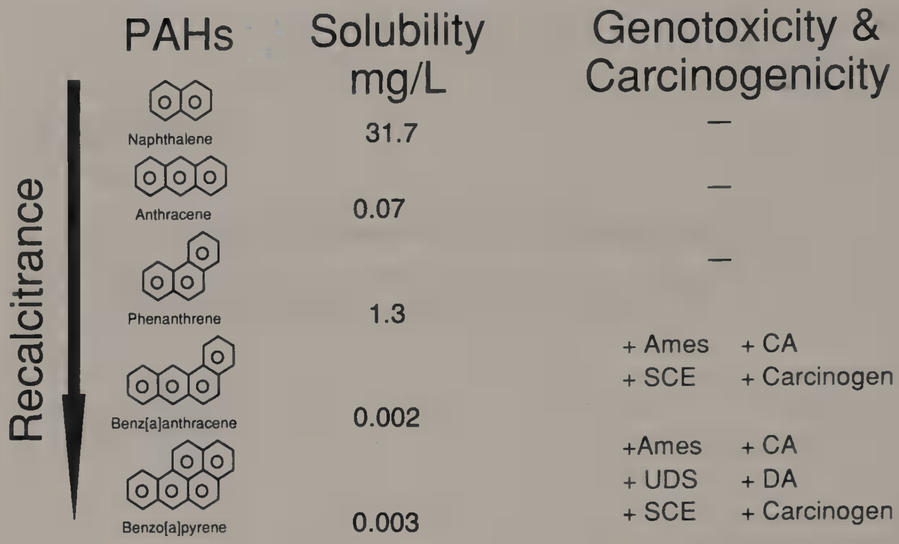
Carl E. Cerniglia

INTRODUCTION

The ability of microorganisms to degrade potentially hazardous organic chemicals has been recognized for many years. In fact, xenobiotic chemicals are potential energy sources for indigenous microorganisms and could be important in the natural cycling of carbon in nature. It has become axiomatic that xenobiotics are susceptible to biodegradation if their chemical structures are similar to those found in naturally occurring compounds. Bacteria and fungi constantly exposed to a structurally diverse range of chemicals have evolved the enzymatic apparatus to degrade a wide variety of organic compounds. Unfortunately, many xenobiotics have structural features never found in naturally occurring compounds and are therefore not readily susceptible to microbial attack.

Polycyclic aromatic hydrocarbons (PAHs) are a major class of environmental contaminants originating from both petrogenic and pyrogenic sources.[1-8] Many PAHs are cytotoxic, mutagenic, and carcinogenic to both lower and higher eukaryotic organisms (Figure 16.1).[2,9-12] Due to their hydrophobic nature, most PAHs in aquatic ecosystems rapidly become associated with particles and are deposited in sediments. A variety of processes, including volatilization, sedimentation, chemical oxidation, photodecomposition, and microbial degradation, are important mechanisms for environmental loss of PAH (Figure 16.2). Microbial degradation of PAH can have a significant effect on the PAH distribution in sediment, especially near the sediment-water interface.[13-16]

There is considerable interest in the use of microorganisms to decontaminate PAH-polluted environments.[17] Successful bioremediation is dependent upon the availability of microorganisms that possess the catabolic enzymes needed to degrade PAH. Mono- and dioxygenases are two groups of enzymes that are


PAHs
Solubility mg/L
Genotoxicity & Carcinogenicity
Recalcitrance
Naphthalene
31.7
—
Anthracene
0.07
—
Phenanthrene
1.3
—
Benz[a]anthracene
0.002
+ Ames
+ CA
+ SCE
+ Carcinogen
Benzo[a]pyrene
0.003
+Ames
+ CA
+ UDS
+ DA
+ SCE
+ Carcinogen

DA = DNA adducts
CA = chromosomal aberrations
Ames = *Salmonella typhimurium* reversion assay
SCE = sister chromatid exchange
UDS = unscheduled DNA synthesis

Figure 16.1. The structures and chemical and toxicological characteristics of polycyclic aromatic hydrocarbons.

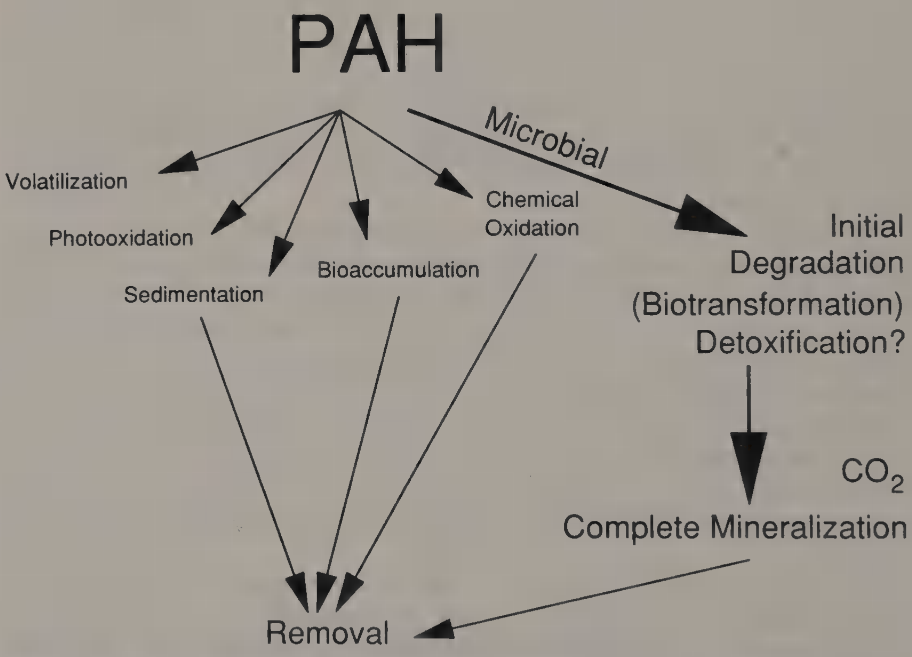

PAH
Microbial
Volatilization
Photooxidation
Sedimentation
Bioaccumulation
Chemical Oxidation
Initial Degradation (Biotransformation) Detoxification?
CO_2
Complete Mineralization
Removal

Figure 16.2. Schematic representation of the environmental fate of polycyclic aromatic hydrocarbons.

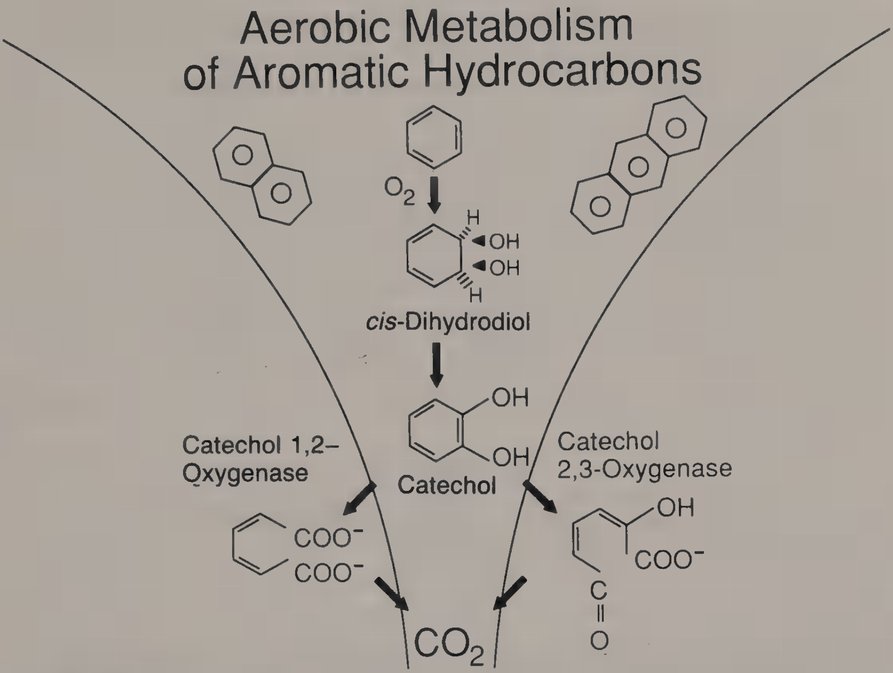


Figure 16.3. Major pathways of bacterial oxidation of polycyclic aromatic hydrocarbons.

important to the microbial catabolism of PAH. Dioxygenases incorporate both atoms of the oxygen molecule into the PAH. This dioxygenase reaction is the major mechanism for the initial oxidative attack on PAH by bacteria, which leads to the formation of dihydrodiols that are in the *cis* configuration.[15] Enzymatic fission of the aromatic ring is also catalyzed by dioxygenases (Figure 16.3). In contrast to bacteria, fungi oxidize PAH via a cytochrome P-450 monooxygenase by incorporating one atom of the oxygen molecule into the PAH and the other into water.[18-23] Metabolic pathways and enzymatic mechanisms for the microbial metabolism of PAHs containing two or three aromatic rings have been well studied.[15] However, there are very few studies on the microbial degradation and detoxification of higher-molecular-weight PAHs. Our current knowledge on the microbial degradation of PAH is summarized below:

1. Biodegradation of lower-molecular-weight PAHs by a wide variety of microorganisms has been demonstrated, and the biochemical pathways have been investigated.[15]
2. There is limited information on the microbial utilization of PAHs containing four or more aromatic rings; however, cometabolism of high-molecular-weight PAHs by bacteria has been demonstrated.[24–32]
3. Biodegradation of unsubstituted PAHs always involves the incorporation of molecular oxygen catalyzed by monooxygenase(s) or dioxygenase(s).[15] How-

ever, there is also increasing interest and speculation concerning anaerobic decomposition of PAH.[33,34]

4. Many of the genes coding for bacterial degradation of PAH are plasmid associated.[35,36]
5. Fungi hydroxylate PAH as a prelude to detoxification, whereas bacteria oxidize PAH as a prelude to ring fission and assimilation.[15,18–23]
6. Fungal metabolism of PAH is highly regio- and stereoselective.[19,22]
7. White-rot fungi have the ability to cleave the aromatic rings of PAH.[37]
8. Microbial degradation of PAH can occur under denitrifying conditions.[33,34]
9. Lower-molecular-weight PAHs, such as naphthalene and phenanthrene, are degraded rapidly in sediments, whereas higher-molecular-weight PAHs, such as benz(a)anthracene or benzo(a)pyrene, are quite resistant to microbial attack.[13,38,39]
10. Environmental factors can have a significant effect on PAH biodegradation.[40]
11. There are higher biodegradation rates for PAH in PAH-contaminated sediments than in pristine sediments.[38,39,41]
12. Procaryotic pathways for naphthalene metabolism predominate in sediments from freshwater and estuarine sediments.[41]

Recent investigations in my laboratory on the biodegradation of PAH has led to the isolation of a *Mycobacterium* sp. that is able to extensively degrade PAHs containing up to five fused aromatic rings.[25,27] The ultimate usefulness of the *Mycobacterium* in the bioremediation of PAH-contaminated sediments depends upon its survival and function in diverse ecosystems.[26] The versatility of the PAH-degrading *Mycobacterium* and its potential for use in the biodegradation of PAH-contaminated sediments will be reported.

MATERIALS AND METHODS

Isolation of the Polycyclic Aromatic Hydrocarbon Degrading Bacterium

The bacterium was isolated from a 500-mL microcosm containing 20 g of sediment, 180 mL of estuarine water, and 100 μg of pyrene.[25,27] The sediment was obtained from a drainage pond chronically exposed to petrogenic chemicals. After incubation of the microcosm for 25 days under aerobic conditions, the sediment samples were serially diluted and screened for the presence of PAH-degrading microorganisms.[25,27]

The screening medium consisted of mineral salts medium (44) containing (per liter): NaCl, 0.3 g; $(NH_4)_2SO_4$, 0.6 g; KNO_3, 0.6 g; KH_2PO_4, 0.25 g; K_2HPO_4, 0.75 g; $MgSO_4 \cdot 7H_2O$, 0.15 g; LiCl, 20 μg; $CuSO_4 \cdot 5H_2O$, 80 μg; $ZnSO_4 \cdot 7H_2O$, 100 μg; $Al_2(SO_4)_3 \cdot 16H_2O$, 100 μg; $NiCl \cdot 6H_2O$, 100 μg; $CoSO_4 \cdot 7H_2O$, 100 μg; KBr, 30 μg; KI, 30 μg; $MnCl_2 \cdot 4H_2O$, 600 μg; $SnCl_2 \cdot 2H_2O$, 40 μg; $FeSO_4 \cdot 7H_2O$, 300 μg: agar, 20 g; and distilled H_2O, 1000 mL.

The surfaces of the agar plates were sprayed with a 2% (wt/vol) solution of a PAH dissolved in acetone: hexane (1:1, vol/vol) and dried overnight at 35°C to volatilize the carrier solvents. This treatment resulted in a visible and uniform surface coat of the PAH on the agar. Inocula (100 μL) from the 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} dilutions of microcosm sediments were gently spread with sterile glass rods onto the agar surface; the plates were inverted and incubated for 3 weeks at 24°C in sealed plastic bags to conserve moisture.

When colonies surrounded by clear zones (Figure 16.4) due to polycyclic aromatic hydrocarbon uptake and utilization were observed (after 2 to 3 weeks), they were subcultured into fresh mineral salts medium containing 250 μg/L each of peptone, yeast extract, and soluble starch, and 0.5 μg/mL of a PAH dissolved in dimethylformamide. After three successive transfers, a bacterium was isolated that was able to degrade pyrene, a PAH containing four aromatic rings.

Growth of Organism and Culture Conditions

The *Mycobacterium* sp. was grown in 125-mL Erlenmeyer flasks containing 30 mL of basal salts medium (19) supplemented with 250 μg/mL each of peptone, yeast extract, and soluble starch and 0.5 μg/mL of pyrene dissolved

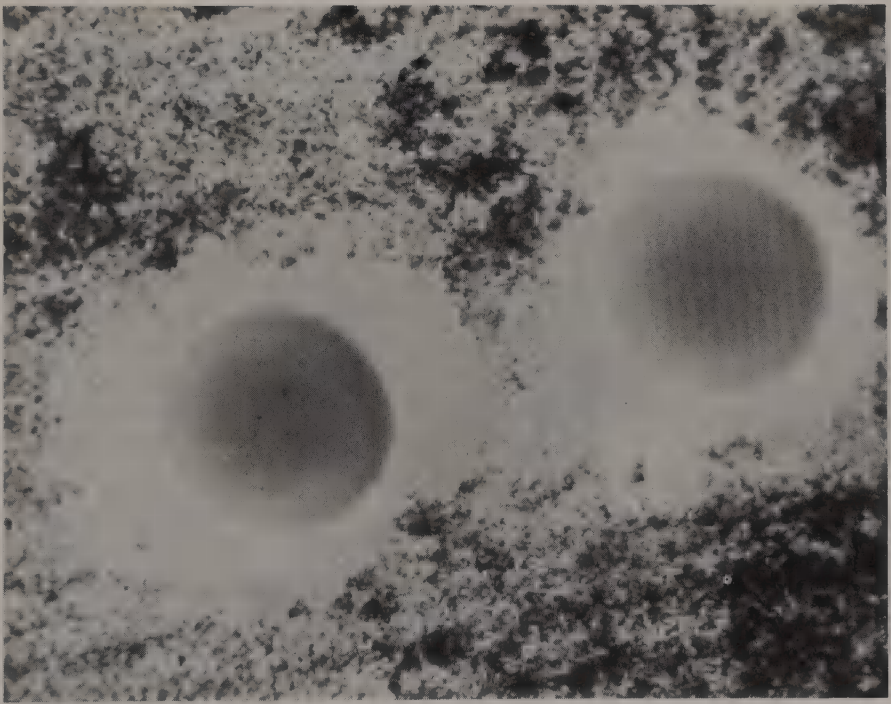


Figure 16.4. Photograph of *Mycobacterium* sp. colonies on MBS agar containing low levels of nutrients and coated with pyrene. The clear zones around the bacterial colonies indicate pyrene utilization.

in dimethylformamide. The cultures were incubated in the dark at 24°C for 72 hr on a rotary shaker operating at 150 rpm. Cells in the midlogarithmic phase of growth were harvested by centrifugation at 8000 G for 20 min at 4°C. The harvested cells were resuspended in sterile 0.1 M *tris*(hydroxymethyl)aminomethane buffer (pH 7.5) at a concentration of 3×10^6 cells/mL and used as inoculum for studies of PAH biodegradation.

Biodegradation Experiments

Biodegradation of PAH by the *Mycobacterium* sp. was monitored in a flow-through microcosm test system.[42–44] This system enables simultaneous monitoring of mineralization (complete degradation to CO_2) and the recovery of volatile metabolites, nonvolatile metabolites, and residual PAH. Microcosms in this test system consisted of 500-mL glass minitanks containing 100 mL of minimal basal salts medium, 0.92 μCi of ^{14}C-labeled PAH, and 50 μg of unlabeled PAH. The PAHs used and their sources were [1,4,5,8-^{14}C]naphthalene (5.10 mCi/mmol), Amersham/Searle Corporation, Arlington Heights, IL; [9-^{14}C]phenanthrene (19.3 mCi/mmol), Amersham/Searle; [3-^{14}C]fluoranthene (54.8 mCi/mmol), Chemsyn Science Laboratories, Lenexa, KS; [4-^{14}C]pyrene (30.0 mCi/mmol), Midwest Research Institute, Kansas City, MO; 3-[6-^{14}C]-methylcholanthrene (13.4 mCi/mmol), New England Nuclear Corporation, Boston, MA; and 6-nitro[5,6,11,12-^{14}C]chrysene (57.4 mCi/mmol), Chemsyn Science Laboratories.

Each microcosm was inoculated with 1.5×10^4 cells/mL, mixed twice weekly, incubated at 24°C for 14 days, and continuously purged with compressed air. The gaseous effluent from each microcosm was directed through a volatile organic trapping column containing 7 cm of polyurethane foam and 500 mg of Tenax GC (Alltech Associates, Inc., Deerfield, IL) and a $^{14}CO_2$ trapping column (50 mL of monoethanolamine:ethylene glycol, 7:3 vol/vol). Mineralization was measured at various intervals by adding duplicate 1-mL aliquots from the $^{14}CO_2$ trapping column to scintillation vials containing 15 mL of a 1:1 mixture of Fluoralloy and methanol (Beckman Instruments Co., Fullerton, CA). Autoclaved inoculated microcosms, and microcosms lacking the *Mycobacterium* sp., were included to detect abiotic PAH degradation.

RESULTS AND DISCUSSION

There are four major objectives in my research program concerning PAH biodegradation:

1. to determine the relationships between chemical structure and PAH degradation by measuring mineralization rates in microcosms, getting good mass balance accountability of undegraded PAH and of volatile and nonvolatile metabolites
2. to isolate microorganisms from environmental sites chronically exposed to

PAHs, which have the ability to degrade PAHs containing four or more aromatic rings

3. to elucidate biochemical pathways and reaction mechanisms for PAH degradation in environmental samples
4. to determine if PAH-degrading bacteria would be useful in the biological decontamination and detoxification of PAH-polluted sites

It is clear from previous investigations that it is relatively easy to isolate microorganisms, using classical enrichment and plating techniques, which can utilize lower-molecular-weight PAHs containing two or three rings. The focus of research in my laboratory is to isolate microorganisms that degrade the higher-molecular-weight PAHs. A summary of our recent investigations is reported below.

Enrichment of PAH-Degrading Bacterium

A pyrene-degrading bacterium was isolated by direct enrichment from sediment samples taken from an oil field near Port Aransas, TX (Figure 16.4). By repeated streaking and isolation, we obtained an isolate, strain Pyr-1, which was identified as a *Mycobacterium* sp. on the basis of the following morphological and biochemical properties:[45] It formed gram-positive, acid-fast rods (1.4 μm in length and 0.7 μm in width). The 15 biochemical tests, mole percent G+C analysis of 66%, and the characterization of the mycolic acids with a carbon chain length of C_{58} to C_{64} were consistent with the assignment of this organism to the genus *Mycobacterium*.

Utilization of PAH by Mycobacterium

The *Mycobacterium* utilized naphthalene, phenanthrene, fluoranthene, pyrene, 3-methylcholanthrene, 1-nitropyrene, and 6-nitrochrysene when grown in mineral salts medium supplemented with low levels of peptone, yeast extract, and soluble starch.[25] This bacterium was unable to utilize these PAHs as the sole source of carbon and energy. Pyrene-induced *Mycobacterium* cultures readily degraded naphthalene (59.5%), phenanthrene (50.9%), fluoranthene (89.7%), pyrene (63.0%), 1-nitropyrene (12.3%), 3-methylcholanthrene (1.6%), and 6-nitrochrysene (2.0%) to CO_2 within 48 hr of incubation (Figure 16.5). Pathways for the initial degradation of pyrene, naphthalene, fluoranthene, and 1-nitropyrene are shown in Figures 16.6–16.9.

The *Mycobacterium* sp. initially oxidized pyrene to form both pyrene *cis*- and *trans*-4,5-dihydrodiols.[28] Oxygen-18 incorporation experiments showed that both atoms of the *cis*-pyrene dihydrodiol were derived from molecular oxygen, but only one atom of molecular oxygen was incorporated into the *trans*-pyrene dihydrodiol (Figure 16.6). 4-Phenanthroic acid, 4-hydroxyperinaphthenone, and cinnamic acid were identified as ring fission products.[28] The *Mycobacterium* sp. initially oxidized naphthalene in the 1,2-positions to form naphthalene-1,2-dihydrodiols. Similar to pyrene oxidation, both the naphthalene *cis*- and *trans*-1,2-dihydrodiols were isolated in a ratio of

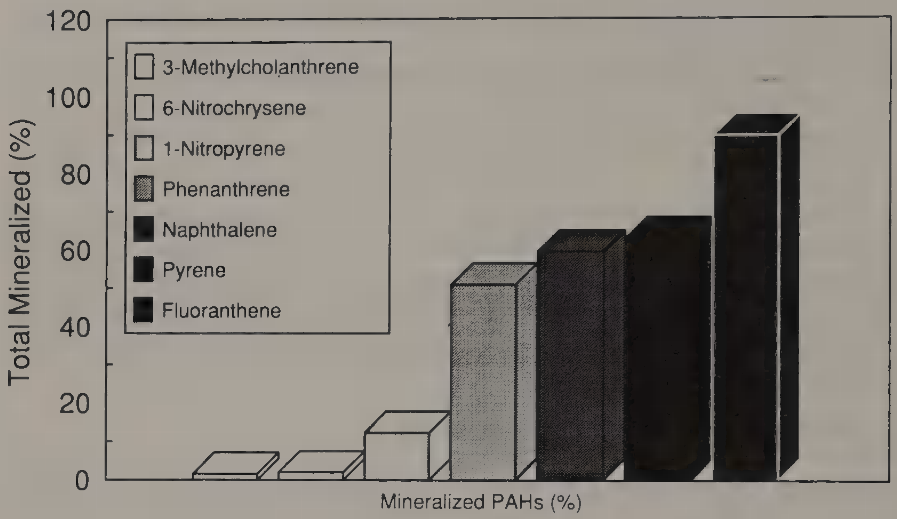


Figure 16.5. Mineralization of 3-methylcholanthrene, 6-nitrochrysene, 1-nitropyrene, phenanthrene, naphthalene, pyrene, and fluoranthene by the *Mycobacterium* sp.

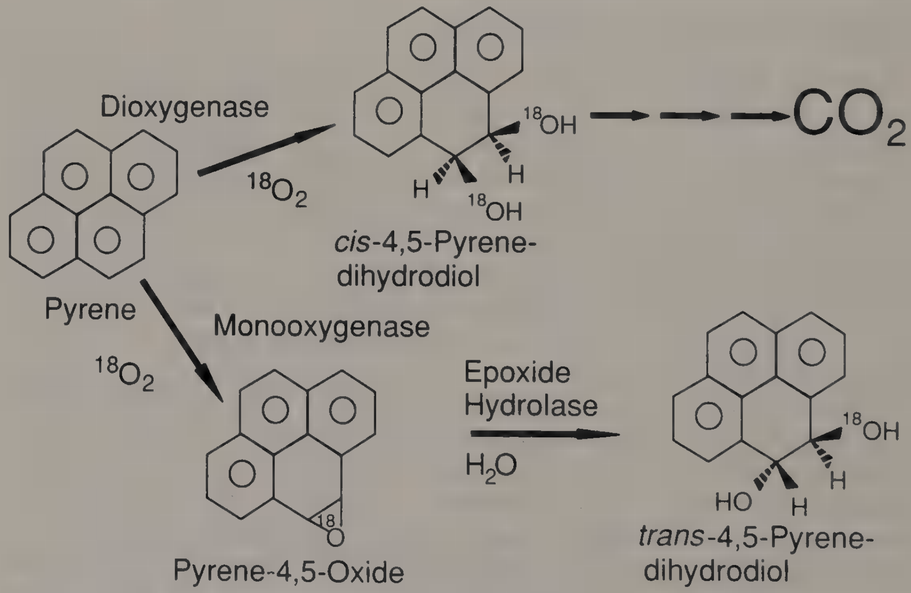

Dioxygenase
$^{18}O_2$
^{18}OH
H
H
^{18}OH
CO_2
cis-4,5-Pyrene-dihydrodiol
Pyrene
Monooxygenase
$^{18}O_2$
Epoxide Hydrolase
H_2O
18
O
Pyrene-4,5-Oxide
^{18}OH
H
HO
H
trans-4,5-Pyrene-dihydrodiol

Figure 16.6. The pathways utilized by the *Mycobacterium* sp. for the oxidation of pyrene.

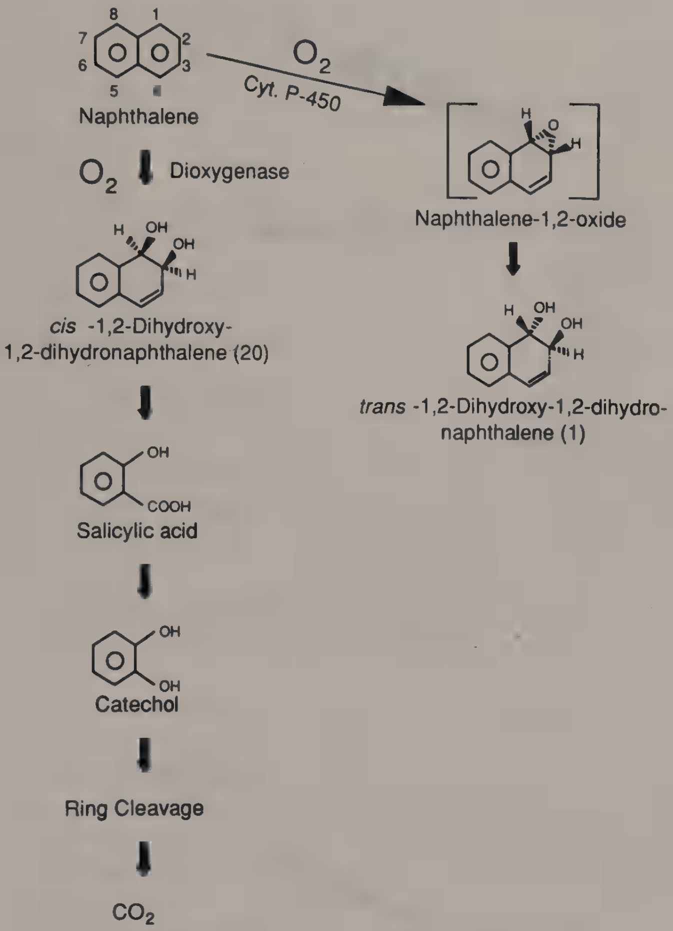

8
1
7
2
6
3
5
4
Naphthalene
O_2
Cyt. P-450
O_2
Dioxygenase
H
O
H
Naphthalene-1,2-oxide
H
OH
OH
H
cis -1,2-Dihydroxy-1,2-dihydronaphthalene (20)
H
OH
OH
H
trans -1,2-Dihydroxy-1,2-dihydronaphthalene (1)
OH
COOH
Salicylic acid
OH
OH
Catechol
Ring Cleavage
CO_2

Figure 16.7. The pathways utilized by the *Mycobacterium* sp. for the oxidation of naphthalene.

20:1. The naphthalene *cis*-1,2-dihydrodiols are further metabolized to salicylate and catechol by the classical bacterial oxidation of naphthalene pathway (Figure 16.7). The *Mycobacterium* sp. extensively degrades fluoranthene to CO_2 (Figure 16.8). However, a ring cleavage metabolite was isolated and identified as 9-fluorenone-1-carboxylic acid. 1-Nitropyrene is degraded very slowly by the *Mycobacterium* sp., and little mineralization occurs, which indicates that the nitro-substituent may sterically block initial enzymatic attack and ring cleavage enzymes since pyrene is rapidly degraded. However, 1-nitropyrene *cis*-4,5-and 9,10-dihydrodiols were isolated and characterized (Figure 16.9).

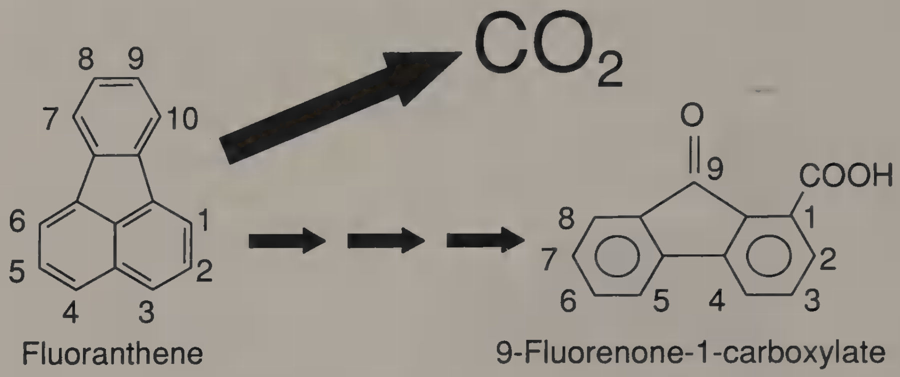

CO_2
8
9
7
10
6
1
5
2
4
3
Fluoranthene
O
9
COOH
8
1
7
2
6
5
4
3
9-Fluorenone-1-carboxylate

Figure 16.8. The pathways utilized by the *Mycobacterium* sp. for the oxidation of fluoranthene.

Microcosm Studies to Evaluate the PAH-Degrading Capacity and Survival of the Mycobacterium When Added to Pristine Sediments

Figure 16.10 indicates that 2-methylnaphthalene and phenanthrene were mineralized to 10 and 14%, respectively, after 28 days in microcosms contain-

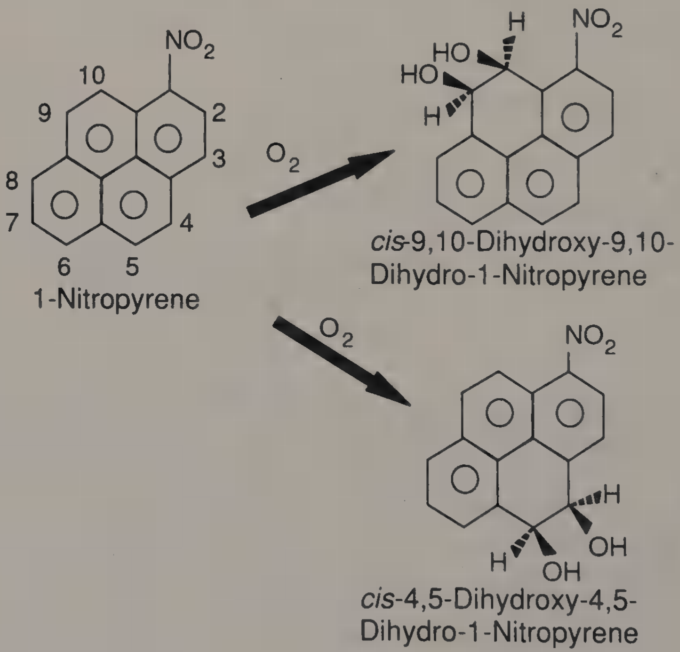

NO_2
10
9
2
3
8
7
4
6
5
1-Nitropyrene
O_2
H
NO_2
HO
HO
H
cis-9,10-Dihydroxy-9,10-Dihydro-1-Nitropyrene
O_2
NO_2
H
OH
H
OH
cis-4,5-Dihydroxy-4,5-Dihydro-1-Nitropyrene

Figure 16.9. The pathways utilized by the *Mycobacterium* sp. for the oxidation of 1-nitropyrene.

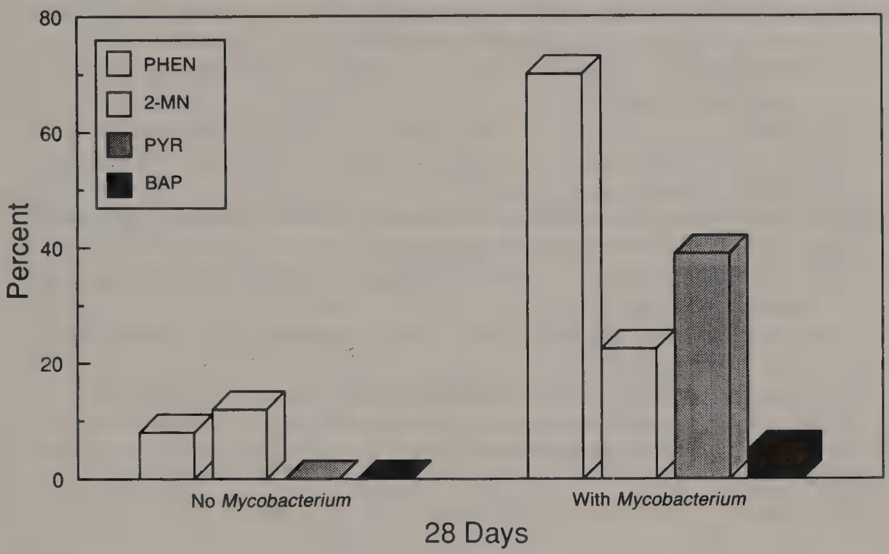


Figure 16.10. Mineralization of phenanthrene *(PHEN)*, 2-methylnaphthalene *(2-MN)*, pyrene *(PYR)*, and benzo(a)pyrene *(BAP)* in microcosms from De Gray Reservoir sediments and water, with and without *Mycobacterium* inoculation.

ing sediment and water from De Gray Reservoir, Arkadelphia, AR. De Gray Reservoir is a pristine lake, which receives relatively little chemical inputs, and has a low PAH-degrading microbial population.[26,38,41] When similar microcosms were inoculated with the *Mycobacterium* sp. (1.5×10^5 cells/g of moist sediment), mineralization of 2-methylnaphthalene and phenanthrene increased to 26 and 71%, respectively. In addition, pyrene and benzo(a)pyrene degradation were observed, whereas previously we did not see degradation of high-molecular-weight PAHs in De Gray Reservoir sediments lacking the *Mycobacterium*. Therefore, the *Mycobacterium* sp. competed with indigenous microflora and enhanced mineralization of PAHs.[26]

Our research indicates that the *Mycobacterium* sp. isolated from an oil-contaminated estuarine site is very versatile and can mineralize low- and high-molecular-weight PAHs. The process is cooxidation, since low levels of organic nutrients are necessary to initiate growth and metabolism of the PAHs. The mechanism of oxidation is unique, since the *Mycobacterium* has both mono- and dioxygenases to catalyze the initial attack on the PAH.

In conclusion, when one discusses the use of microorganisms in the remediation of hazardous wastes, such as PAH, some bioremediation issues that should be addressed are the following:

1. a complete understanding of the chemical, toxicological and ecological characterization of the site
2. more data on the fate, metabolism, and kinetics of high-molecular-weight PAH biodegradation at the site

3. determination of biochemistry and mechanisms of many of the high-molecular-weight PAH degradative pathways
4. determination of conditions that will ensure the survival of the biological detoxification system
5. effective transportation of the biological detoxification system to the site
6. development of procedures for employing immobilized cells to decontaminate PAH-contaminated soils
7. determination of whether bioremediation is a cost-effective means of cleanup of PAH-contaminated wastes
8. getting the PAH-degrading microorganisms (large biomass) there and making them grow and function
9. understanding the fate of plasmid DNA or recombinant strains in wastewater or sediments
10. optimizing a PAH-degrading microbial system for environmental use
11. basic research on coupling aerobic and anaerobic biodegradation systems
12. research on specific bacteria used at a site, such as salt-tolerant or chemical-tolerant bacteria

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CHAPTER 17

The Use of Chemical Diffusing Substrata to Monitor the Response of Periphyton to Synthetic Organic Chemicals

Scott D. Schermerhorn, Gina Abbate, and Roy M. Ventullo

INTRODUCTION

The role of naturally occurring bacterial communities in the removal of chemicals from aquatic systems is being addressed.[1-4] Of special interest are those communities of bacteria that attach to a substratum and form a complex community with algae and fungi, often referred to as *periphyton*.[5-7] Periphyton can be found attached to rock, cobble, and other stationary surfaces in most flowing water systems. Because of the close physical proximity of the heterotrophic and autotrophic components, efficient cycling of CO_2, O_2, carbon, and nutrients may occur between trophic levels.[6-8] It has also been suggested that the heterotrophic components within periphyton communities play a major role in the biodegradation of allochthonous organic input.[2,9]

Laboratory studies are often used to measure rates of biodegradation, which are then used to estimate degradation in the environment. It is often difficult to extrapolate results obtained in laboratory systems to project the fate of chemicals in the environment, where conditions may be, and often are, different from those in the laboratory. The effects of many parameters need to be considered: temperature, nutrient concentration, chemical structure and concentration, as well as the acclimation of the microbial community to biodegrade the compound of interest. Chemical fate models need accurate information about biodegradation in situ to more closely predict the fate of both natural and xenobiotic chemicals in aquatic systems.[10,11]

In situ bioassay systems that have used point-source manipulation of inorganic nutrients have been successful in influencing communities in lotic and benthic environments.[12,13] Until recently, only inorganic manipulation of the autotrophic component of the periphyton community through point-source diffusion has been reported.[12-15] The advantages of point-source diffusion through substrata are the following:

1. Concentrations of material diffusing outward can be measured and controlled while the community is in its ever changing natural environment.
2. The substrata can provide relatively high chemical concentrations to the periphyton communities while adding little to the large volume of the stream.
3. Treatments using this technique can be replicated and placed in a variety of different areas within an aquatic system.

Periphyton growth on such substrata should be influenced by the release of material (inorganic or organic), and the results of this influence can be examined both quantitatively and qualitatively with regard to biomass, bacterial and algal numbers, primary and secondary production, and biodegradative ability.

Since it has been suggested that acclimation to chemicals is an important process regulating biodegradation rates,[3,10,16] our goals were

1. to develop a bioassay system in which a diffusing substratum would serve as a point source of synthetic organic chemical release
2. to use such a system to measure periphyton response to constant chemical exposure in situ
3. to characterize acclimation periods and biodegradation kinetics of synthetic organic chemicals by the periphyton communities

MATERIALS AND METHODS

Sample Site

Periphyton communities were obtained by incubating the bioassay system in the East Fork of the Little Miami River, east of Cincinnati, Ohio. Periphyton communities were allowed to colonize and develop for 3 weeks.

Organic Chemicals

Six organic chemicals were examined in this study: linear alkylbenzene sulfonate (LAS), nitrilotriacetic acid (NTA), linear primary alcohol ethoxylate (LAE), 4-nitrophenol (PNP), Monotallowtrimethylammonium chloride (MTTMAC), and a detergent builder, succinate tartrates (ST). All chemicals were obtained from the Procter and Gamble Company (Cincinnati, OH), with the exception of 4-nitrophenol (Aldrich, Milwaukee, WI). Radiolabeled materials (^{14}C) used for the mineralization studies were obtained from Procter and Gamble and were shown to be greater than 98% radiochemically pure by HPLC and/or TLC analysis.

Organic Chemical Diffusion Studies

The six organic chemicals were individually added to solutions of 20% molten agar to obtain final concentrations of either 20 or 200 mM. Trace amounts of radiolabeled chemical were added to each solution in a ratio that allowed for the detection of 1% release. The solutions were then mixed well, poured into ceramic saucers, and allowed to solidify. The tops of the saucers were sealed with Plexiglas and silicone sealant. The diffusing ceramic substrata were then placed in glass bowls filled with distilled water, which was continually stirred. Water from the glass bowls was removed daily, assayed for radioactivity by liquid scintillation spectrometry, and replaced with the same volume of fresh distilled water for a minimum of 21 days.

Inorganic Diffusion Studies

Nitrate and phosphate, as $NaNO_3$ and K_2HPO_4, were individually added to solutions of 20% molten agar to achieve final concentrations of 500, 50, or 20 mM. The solutions were poured into ceramic substrata, allowed to solidify, and sealed as described above. The substrata were then placed in 18 megohm ultrapure water (Millipore Corporation), which was continually stirred. Water was removed daily and assayed for NO_3^- and PO_4^- ions by ion chromatography (Dionex Corporation). A Series 2000 ion chromatograph with an HPIC/AS4 strong-anion exchange column was used with 0.25 mM carbonate eluent at a flow of 2 mL/min, and 0.025 N H_2SO_4 served as the regenerant. Anions were quantified by peak area using an SP 4620 integrator. Calibration was by Dionex standard 5-anion solution (Dionex Corporation).

Exposure Studies

Clay ceramic saucers with a known surface area (141.5 cm^2) were filled with agar solutions containing various concentrations of LAS, LAE, and ST, and were attached to a 4-ft × 4-ft sheet of Plexiglas. In a second experiment involving low concentrations of LAS, inorganic nitrate (500 mM) and phosphate (20 mM) were also added. The Plexiglas was then suspended under a rack of PVC pipe and polyfloats and placed in the river. The ceramic substrata were positioned within the photic zone of the stream at a depth of 18 in. below water surface. This system was modeled after other floating in situ assay systems.[14,17,18] Periphyton communities were allowed to develop for at least 21 days on the ceramic substrata placed in the East fork of the Little Miami River. The substrata were recovered, and a portion of the periphyton communities (10 cm^2) was carefully scraped into 125-mL flasks and equipped with CO_2 traps. Samples were spiked with appropriate ^{14}C substrate (5–8 μCi, 50 μg/L), and the $^{14}CO_2$ released due to mineralization/respiration of the test compound was trapped with 0.75 N KOH and counted by liquid scintillation spectrometry.[1,16] Data were expressed as the cumulative percentage of the radiolabeled compounds recovered as $^{14}CO_2$.

Biodegradation Parameters

Mineralization curves were fitted to a first-order production equation:[1,16]

$$y = a(1 - e^{(-k(t-c))})$$

where y = percent of initial ^{14}C added recovered as $^{14}CO_2$
t = time in days
a = the extent of degradation (asymptote of CO_2 produced)
k = the first-order rate constant
c = the lag time before the onset of mineralization

This equation provides the kinetic parameters of the first-order rate constant, mineralization extent, mineralization half-life (ln 2/k), and lag-time. All parameter estimates (a, k, c) were obtained by least-squares analysis by using iterative techniques and a nonlinear computer program (Statpro, Penton Software).

Structural Characteristics

Periphyton communities were also analyzed for structural components. Protein and carbohydrate concentrations were determined spectrophotometrically on homogenized communities.[19,20] Dry weights, as well as total numbers of bacteria, determined by direct fluorescence microscopy, were also performed.[21]

Statistical Analyses

Statistical analyses were performed by nonparametric techniques using a computer program (Statpro, Penton Software). Nonparametric statistical analyses were used because of the limited number of true replicates ($N = 2$ to 5 individual replicates) and the lack of a normal distribution on which to base parametric assumptions. Variance was determined by Kruskal-Wallis (nonparametric ANOVA) and Mann-Whitney U tests performed on all true replicates. Significance levels were determined based upon tables compiled by Rohlf and Sokal.[22]

RESULTS

Diffusion Studies

All organic compounds tested diffused out of the ceramic substrata at a measurable rate. These laboratory studies indicate that the rate of release for the chemicals was on the order of micrograms per day (Figure 17.1). LAS diffused at a mean rate of 1.5 $\mu g/cm^2/day$ from an internal concentration of 200 mM. The 200-mM solutions of LAE, NTA, and ST were released at a mean rate of 365 $\mu g/cm^2/day$, 34.6 $\mu g/cm^2/day$, and 113 $\mu g/cm^2/day$, respectively. MTTMAC was released at a mean rate of 58.3 $\mu g/cm^2/day$. Release rates for PNP were 5 $\mu g/cm^2/day$ and 1 $\mu g/cm^2/day$ for the two concentrations examined. LAE (a nonionic water-soluble compound), NTA (a low-

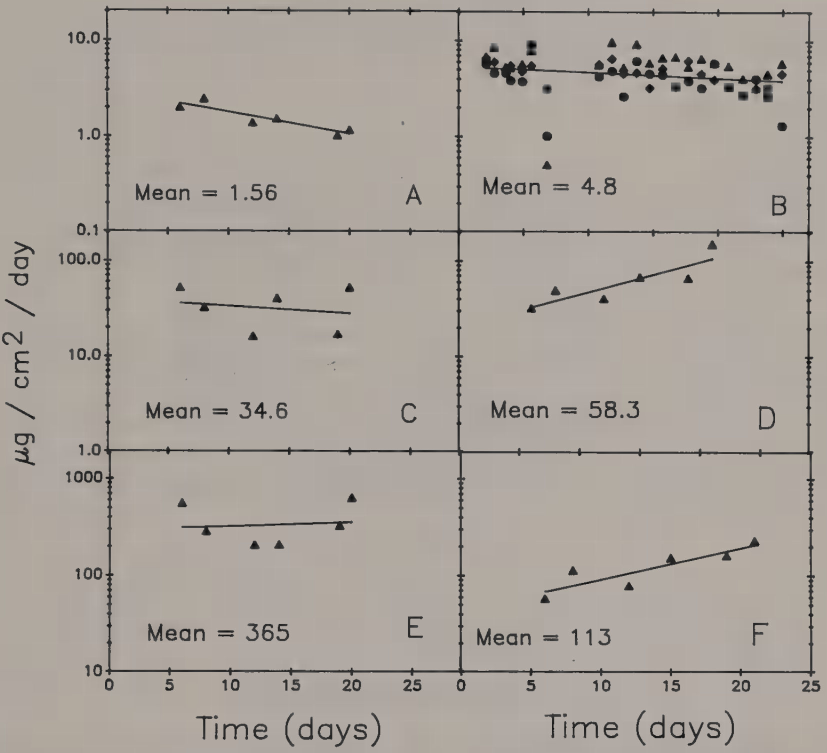

Figure 17.1. Diffusion of various synthetic organic chemicals through ceramic substrata (n = 3): *(A)* LAS, *(B)* PNP, *(C)* NTA, *(D)* MTTMAC, *(E)* LAE, and *(F)* ST. Internal concentration for all chemicals was 200 mM. All data points shown are mean values (n = 3) of replicate substrata except *B*, which illustrates the reproducibility of the diffusion rates through replicate substrata (▲, ●, ◆).

molecular-weight but charged compound), and MTTMAC (a quaternary ammonium compound) all diffused more readily than LAS (a negatively charged hydrophobic compound) and PNP (a substituted phenol). The results indicate that these chemicals are available to the communities on the outside surface, and the amount released is proportional to the amount added to the dish. It appears that hydrophobicity, molecular structure, and charge regulate diffusion rates.

The diffusion of inorganic chemicals was also measured. As expected, release rates for NO_3 and PO_4 demonstrated dependence on internal concentration. Mean release rates for NO_3 and PO_4, over a 30-day period from an internal concentration of 500 mM, were 40 and 1200 $\mu g/cm^2/day$, respectively. The release of PO_4 was undetectable from internal concentrations of 20 mM. Release rates of NO_3 and PO_4 declined over time and were within the range of previously reported values.[12–15,18] We also detected the release of SO_4 at a rate of 33 $\mu g/cm^2/day$. Since no sulfate salts were added, the likely source was the ceramic substrata and/or the agar. These sources provided SO_4 to the communities from all substrata, including unamended controls.

Exposure Studies

Having established that these various organic compounds were released and available to the outer surface of the ceramic substrata at predictable concentrations, we employed an in situ bioassay system to measure the effect of chemical exposure on periphyton communities. The periphyton was exposed to various concentrations of unlabeled chemicals for 21 days. Exposure of the community resulted in shorter lag times and a greater extent of mineralization than seen in the unexposed controls (Figures 17.2 and 17.3). For unexposed communities, the mean lag-time for LAS was 10 hr, the maximum percentage mineralized was 38%, and the half-life was 76 hr. Those communities previously exposed to LAS degraded the chemical faster (half-life, 40 hr) and to a greater extent (60–70% CO_2) with no appreciable lag period evident (Figure 17.2).

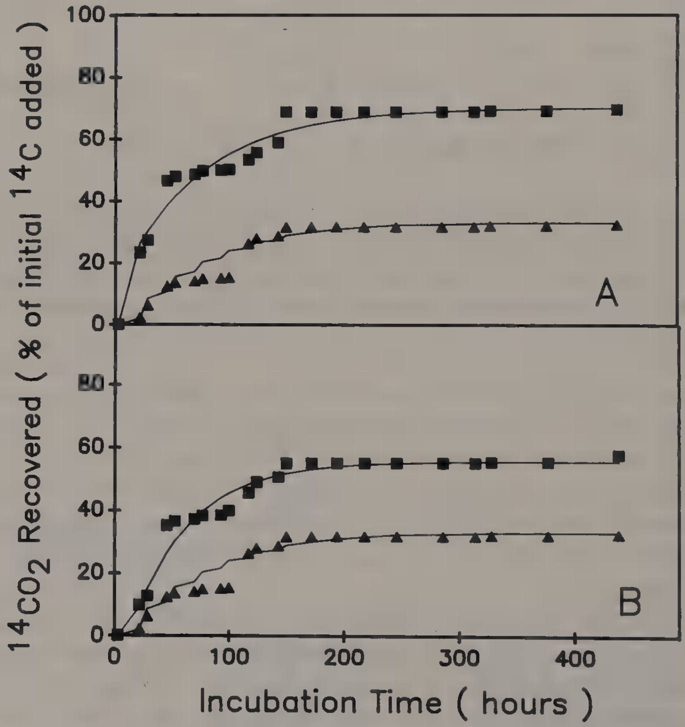

Figure 17.2. Percent of radiolabeled LAS recovered as $^{14}CO_2$ in representative periphyton communities exposed to *(A)* less than 0.1 μg LAS/cm²/day (▲, control; ■, exposed) and *(B)* 1.56 μg LAS/cm²/day (▲, control; ■, exposed). Data points plotted around first-order mineralization curve as predicted by nonlinear regression.

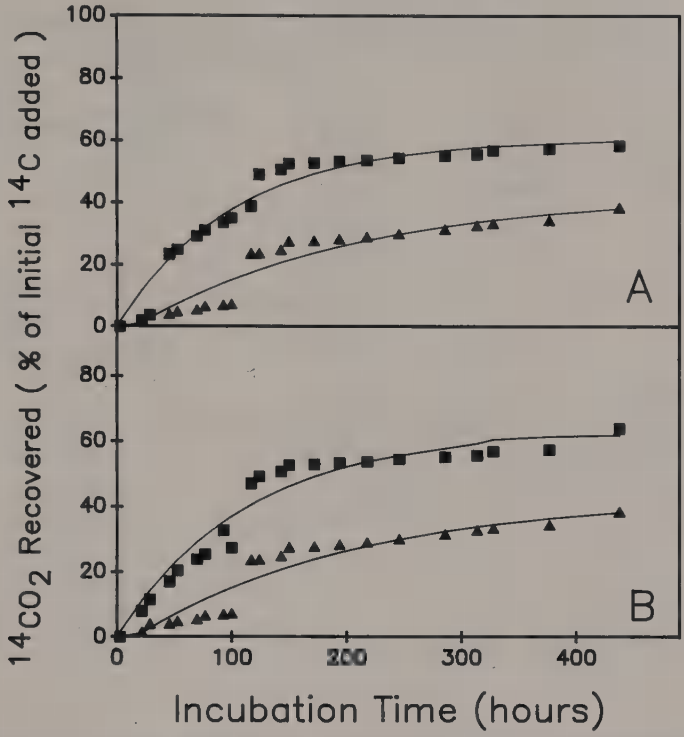

Figure 17.3. Percent of radiolabeled ST recovered as $^{14}CO_2$ in representative periphyton communities exposed to *(A)* 113 μg ST/cm²/day (▲, control; ■, exposed) and *(B)* 1.13 μg ST/cm²/day (▲, control; ■, exposed). Data points plotted around first-order mineralization curve as predicted by nonlinear regression.

The mineralization of succinate tartrates yielded similar results. In general, the unexposed communities showed longer lag times and half-lives when compared to those communities that were exposed. Lag time was reduced by 10 hr in those communities that were exposed to ST. The extent of mineralization ranged from 48–55% in all communities. Communities that were exposed to 11 μg/cm²/day mineralized more, and had a shorter half-life (67 hr), than the unexposed communities (Table 17.1).

LAE degradation by periphyton communities was rapid and extensive. However, exposure to the chemical had little effect on the kinetics of mineralization (Table 17.1). The mean half-life for periphyton communities, both exposed and unexposed to LAE, was 2 days, and approximately 80% was mineralized (Figure 17.4). Previous studies on the biodegradation of LAE have also reported rapid and extensive mineralization of the compound.[23]

To determine if chronic exposure of the periphyton to lower concentrations of a chemical would elicit similar responses in biodegradation kinetics, a second experiment was carried out using linear alkylbenzene sulfonate released at concentrations of ng/cm²/day. Those communities that were exposed to

Table 17.1. Biodegradation Kinetic Parameters for Periphyton Exposed to Various Synthetic Organic Chemicals

Chemical Exposure (μg/cm^2/day)		Mean Rate Constant (day^{-1})	Mean Lag Time (day^{-1})	Mean Half-Life days	Mean $^{14}CO_2$ Evolved (% of ^{14}C added)
LAE	0.0	0.35 ± 0.07	0.14 ± 0.14	2.0	79
	360	0.33 ± 0.07	0.00	2.1	77
ST	0.0	0.22 ± 0.12	0.62 ± 0.33	3.1	40
	11	0.25 ± 0.02	0.28 ± 0.07	2.8	55
	113	0.17 ± 0.09	0.19 ± 0.19	4.0	50
LAS	0.0	0.22 ± 0.05	0.37 ± 0.07	3.2	38
	0.15	0.42 ± 0.03[a]	0.05 ± 0.06[a]	1.6[a]	68
	156	0.40[a] ± 0.01[a]	0.28 ± 0.04[b]	1.7[a]	60

Note: Mean values (n = 2 to 3) ± standard deviation.

[a] Significantly different from unexposed control at $p<0.05$ level.

[b] Significantly different from control at $p<0.10$ level.

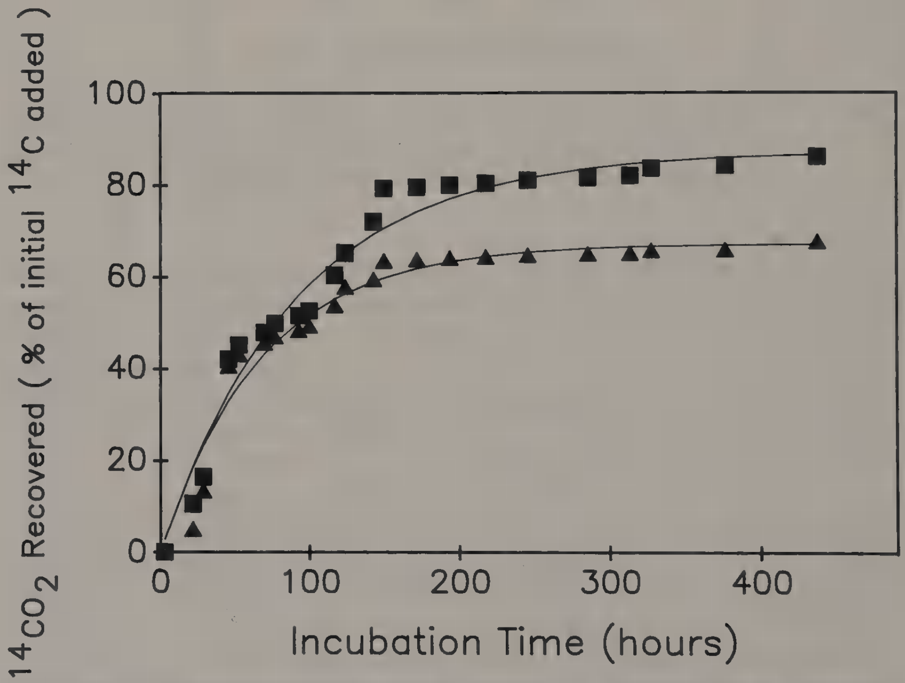

Figure 17.4. Percent of radiolabeled LAE recovered as $^{14}CO_2$ in representative periphyton communities exposed to 365 μg LAE/cm^2/day (▲, control; ■, exposed). Data points plotted around first-order mineralization curve as predicted by nonlinear regression.

Table 17.2. Kinetic Parameters for Periphyton Communities Exposed to Linear Alkylbenzene Sulfonate

LAS Exposure ($ng/cm^2/day$)	Rate Constant (day^{-1})	Lag Time (day^{-1})	Half-Life (days)	$^{14}CO_2$ Evolved (% of ^{14}C added)
0.0	0.14 ± 0.04	0.41 ± 0.07	5.4	50 ± 16
0.15	0.22 ± 0.04	0.40 ± 0.05	3.6	72 ± 4
15	0.20 ± 0.02[a]	0.30 ± 0.04[a]	3.4[a]	82 ± 1
15[b]	0.22 ± 0.02[a]	0.26 ± 0.03[a]	3.2[a]	63 ± 6
150	0.21 ± 0.02[a]	0.29 ± 0.03[a]	3.2[a]	77 ± 10

Note: Mean values (n = 3 to 5) ± standard deviation.
[a] Significantly different from the unexposed controls at $p<0.05$ level.
[b] Exposed to 40 μg $NO_3/cm^2/day$.

15–150 ng LAS/cm²/day differed significantly in rate constant, lag time, and half-life from the unexposed controls (Table 17.2). The same communities did not differ statistically with regard to bacterial numbers or dry weight (Table 17.3). Periphyton communities exposed to less than 1 ng/cm²/day exhibited no differences from the unexposed controls. The communities that were exposed to 15 ng LAS/cm²/day in combination with 40 μg $NO_3/cm^2/day$ also showed significant differences in rate constants and half-lives when compared to the unexposed controls, but not from those communities exposed to LAS alone.

DISCUSSION

The diffusion rates of organic chemicals through the ceramic substrata were constant, showing only slight decline over an extended time period. As has been demonstrated for inorganic nutrients, the diffusion of organic material was linear, and the amount of chemical influencing the periphyton community could be estimated. Organic chemical release rates did not appear to decline over time as precipitously as reported for inorganic ions.[12,14,17] This may have been due to the characteristics of the organic compounds tested. The organic chemicals are larger molecules and perhaps more interactive with the ceramic and/or the agar than inorganic ions. It is evident, however, that the internal

Table 17.3. Structural Characteristics of Periphyton Communities Exposed to Linear Alkylbenzenes Sulfonate

LAS Exposure ($ng/cm^2/day$)	Dry Weight (mg/cm^2)	Bacteria ($\times 10^7$ cells/cm^2)	Protein ($\mu g/cm^2$)	Carbohydrate ($\mu g/cm^2$)
0.0	15.5 ± 2.8	5.31 ± 1.54	588 ± 142	617 ± 181
0.15	16.0 ± 2.7	3.69 ± 1.29	927 ± 529	517 ± 238
15	10.7 ± 1.8	4.95 ± 0.94	588 ± 378	336 ± 254
15[a]	12.1 ± 5.0	6.55 ± 1.54	183 ± 214	259 ± 55[b]
150	9.3 ± 2.8	5.00 ± 0.86	216 ± 72[b]	296 ± 136[b]

Note: Mean values (n = 3 to 5) ± standard deviation.
[a] Exposed to 40 μg $NO_3/cm^2/day$.
[b] Significantly different from the unexposed controls at $p<0.01$ level.

chemical concentration can be set far enough over the threshold concentration needed to influence the community (discussed below) as to make the decline in chemical release over time negligible.

The nature of the chemical tested influenced the release rate (Figure 17.1). When the internal concentrations of the chemicals were equal (200 mM), the differences in release rates were likely dependent on molecular structure, molecular weight, hydrophobicity, and charge. The compounds with the highest (LAE) and the lowest (LAS) release rates are similar in molecular weight. The differences in the rate of release can be attributed to the nonionic and hydrophilic nature of LAE. These properties resulted in a less sorptive molecule compared to LAS, which is negatively charged with a hydrophobic alkyl chain.

It has been shown that the prior exposure of bacterial communities to synthetic organic chemicals reduces the lag time before mineralization.[1,2,4,9,24] This study confirms results obtained by other investigators. In addition to shortened lag times, increases in the mineralization rate constants were also observed (Tables 17.1 and 17.2). Explanations for such differences are being explored. Several hypotheses have been offered to explain lag times:

1. the time for microbial populations to grow to a sufficient size to affect the removal of the compound
2. induction or derepression of enzymes necessary for degradation
3. genetic change, i.e., mutation, gene exchange, or rearrangement
4. diauxie patterns of preferential substrate usage

Other work has suggested that an increased number of organisms is responsible for the greater activity.[25–27] Additional explanations that have been suggested include the absence of essential inorganic nutrients, transient inhibitory conditions, predation by protozoa, and the nature of the chemical.[3,9] In our study, physical and chemical parameters were maintained at natural conditions with only the presence of the chemical varied, thereby eliminating other environmental factors as an influence on the acclimation time before mineralization. The release of chemicals in this study was low, on the order of $\mu g/cm^2/$ day, and was likely below the threshold for bacterial growth as a sole substrate.[26,28] We postulate that the cells were induced by the presence of the trace concentrations of the chemical but grew on photosynthate in the periphyton matrix and/or autochthonous dissolved organic carbon in the water column of the stream.

Initial rates of chemical release may influence the types of pioneering organisms that adhere and colonize the substrata. The ceramic substrata tested also provided a selective surface for populations that were resistant to any toxic effects of the compound being released. Further, communities chronically exposed to chemicals may be specialized in the use of that compound for energy and carbon requirements. Such specific heterotrophic assemblages in the community may outcompete other components of the periphyton for nutrients or adherence sites, or use extracellular secretions that may result in a low species diversity.

Rate constants and half-lives varied between the two experiments involving LAS (Tables 17.1 and 17.2). Such variability may be attributed, in part, to seasonal differences. Rate constants were higher and half-lives shorter in those samples colonized in late summer than in those colonized in winter (mid-December). Inorganic NO_3 did not stimulate LAS biodegradation, probably due to the already high concentration of NO_3 found in the water column (~ 7 mg/L). Inorganic PO_4 likewise had no effect and can be assumed not to have influenced the periphyton communities since the internal concentration used resulted in no detectable release in laboratory diffusion studies (above). Since bacterial density, as well as biomass estimates of protein, carbohydrate, and dry weights, were similar for all exposed and unexposed communities (Table 17.3), our results indicate that some type of acclimated population was present on all substrata that released chemical compound. It is not known if specific degraders capable of mineralizing the diffusing compound were the first to colonize these substrata and reproduced, or if the initial colonizers underwent some type of genetic regulation/deregulation.

Communities exposed to succinate tartrates also demonstrated similar trends of shorter half-lives and lag times, supporting the notion that the heterotrophic component of the epilithic community was influenced by the presence of the chemical. As has been previously demonstrated, LAE is a readily degradable compound, and populations need not acclimate to it in order for biodegradation to occur.[23] Since acclimation by periphyton to some chemicals occurs, rate data must incorporate acclimation time for modeling chemical fate in aquatic systems.[2,3,10,11] Only by determining the lag time for active degradation can estimations of bioremoval be used to enhance the predictability of chemical fate modeling.

The ceramic substrata incubated in situ can serve as a reliable point source of material to study periphyton response to organic and inorganic amendment. Such a system allows acclimation to occur in situ and has potential for use in a variety of studies, including the response to nutrient stimulation,[13,14,18] exploration of diauxie, and toxicity assessments. Such a system can also be used to determine biodegradation kinetics of other synthetic organic compounds in situ without exposing the environment to high concentrations of chemical. In addition to aquatic settings, ceramic substrata may be useful as a point source of chemicals in sediments.

CONCLUSIONS

Several conclusions can be drawn from this study:

1. Organic compounds of various structure and charge diffuse through ceramic substrata.

2. Periphyton communities grown on such diffusing substrata are influenced by the material released.
3. In response to chemical diffusion, first-order biodegradation rates and the extent of mineralization are increased.
4. Lag time preceding mineralization is decreased by the preexposure of communities to those compounds.
5. The in situ bioassay system which capitalizes on point-source diffusion is a useful system to study the response of periphyton communities to synthetic organic chemicals.

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C. Genetic Engineering and Molecular Techniques

CHAPTER 18

Molecular Analysis of Biodegradative Bacterial Populations: Application of Bioluminescence Technology

Gary S. Sayler, J. M. Henry King, Robert Burlage, and Frank Larimer

INTRODUCTION

Subsurface soil contamination and groundwater pollution problems have stimulated considerable attention concerning the potential for in situ bioremediation as an efficient and cost-effective treatment technology. This attention is directed toward utilizing the degradative capacity of bacteria to eliminate specific contaminants from the subsurface environment. Yet, it has been only in the past decade that the occurrence of relatively diverse and viable microbial populations in shallow aquifers[1,2] and deep subsurface materials[3,4] has been demonstrated and fully appreciated.

The potential for in situ bioremediation of subsurface contamination is limited, in a practical sense, to two general approaches: (1) stimulation of degradative activity of indigenous bacterial populations and (2) introduction of nonindigenous microorganisms with known degradative potential.

The first approach assumes that the necessary genetic information encoding specific biodegradative pathways or cometabolic degradation (such as methanotrophic oxidation of trichloroethylene) exists within the subsurface microbial community. The second approach assumes that the necessary genetic information can be introduced and maintained in the subsurface microbial community sufficiently long to accomplish the remediation objectives. In this regard, subsurface bioremediation is no different than other bioremediation practices or biological waste treatment. However, due to the occluded and often remote nature of the contaminants in this environment, the application of reliable engineering strategies for the remediation process and monitoring of the biological activity is difficult. Consequently, methods to enhance the population density and metabolic activity of degradative organisms (whether indigenous or not) are confined to the addition of nutrients and electron acceptors, control of hydraulic flow rates, and optimizing partial pressure of gaseous components (such as O_2 and CH_4) in the system.

The success of such bioremediation efforts ultimately is determined by the genetic capacity for biodegradation. Relatively little work has been done to evaluate the occurrence of known genetic systems mediating biodegradation or to apply molecular biological techniques to evaluate the genetic information or gene products associated with biodegradation in subsurface environments. An objective of this chapter is to summarize current molecular information on the occurrence of bacterial plasmids and specific degradative (catabolic) genes in subsurface microbial communities. A second objective is to demonstrate the development and use of biomolecular techniques such as bioluminescent reporter technology to measure activity of degradative genes in subsurface materials.

PLASMIDS IN SUBSURFACE BACTERIA

Plasmids are extrachromosomal genetic elements existing as closed circular double-stranded DNA molecules. They are relatively small, 0.1–1.0% the size of the bacterial chromosome, but may contain all the genes necessary for complete catabolism of some pollutants, such as toluene, naphthalene, and chlorobiphenyl. While degradative genes are not unique to plasmids, a wide variety of bacterial species maintain plasmids encoding a diversity of degradative activities.[5] In some polluted environments the frequency of bacteria carrying plasmids may approach 50% and is positively correlated with pollution.[6–8]

Three studies have examined the distribution of plasmids in subsurface bacteria: one in shallow aquifers[8] and two in deep subsurface sediments[9,10] (Table 18.1). In these studies, bacterial populations were prevalent in most samples at densities above 1×10^4/g and up to 1×10^8/g, even in deep subsurface core materials. Plasmids were abundant in nearly all sample materials and were found at a significantly higher frequency (by a factor of two) in deep subsurface materials than in shallow aquifer material. It was also noted that the plasmids of many of the deep subsurface bacteria were quite large, often exceeding 400 kb (kilobase pairs), while plasmids from shallow aquifers rarely exceeded 100 kb in size. This may be partly attributed to differences in plasmid isolation techniques.[9] In general, most catabolic plasmids are larger than 50 kb, and the conclusion can be drawn that many of these plasmids are large enough to contain catabolic function.

The presence of DNA sequences related to the toluene catabolic plasmid TOL (117 kb) was examined in all three studies,[8–10] using different forms of DNA hybridization detection. No TOL-related sequences were found in the bacterial populations from shallow aquifers;[8,10] however, seven bacterial isolates were recovered from the deep subsurface that had sequence homology to TOL,[9] four of which appeared to be plasmid associated. Jimenez also detected

Table 18.1. Comparative Plasmid Distributions in Subsurface Bacterial Populations

Sample Type	(n)	Contamination	Depth (m)	Bacterial Density $g^{-1} \times 10^4$	Plasmid Frequency %	Toluene[a] Plasmid	References
Shallow Aquifer	(6)	none	4–6	.5–9.1	3.2	–	Ogunseitan et al.[8]
	(5)	contaminated	7–8.5	5–9.5	17.5	–	
Deep Subsurface	(50)	none	0.2–260	<.001–10,000	37.8	+	Fredrickson et al.[9]
Deep Subsurface	(8)	none	21–420	.1–1,000	nd	+	Jimenez[10]

[a] Presence of DNA homologous to TOL plasmid DNA as determined by either colony, southern, or blot hybridization with TOL plasmid DNA. (nd, not determined) (n, number of samples).

TOL sequences in total DNA extracts from the same deep subsurface materials.[10]

The TOL plasmid has been introduced successfully into shallow aquifer microbial communities under laboratory simulation.[11] A strain of *Pseudomonas putida* harboring the TOL plasmid and an antibiotic resistance plasmid RP4 (60 kb) was maintained up to 8 weeks in aquifer material at cell densities of approximately 1×10^4/g.[11] Using DNA hybridization procedures, cells harboring both of these plasmids could be readily discriminated from the indigenous bacteria in the sample.[11] Similar DNA hybridization techniques were useful for monitoring the population density of indigenous bacterial strains in the aquifer samples.[11,12] In addition, a newly described species, *Pseudomonas geomorphus,* indigenous to the shallow aquifer environment was found to accept and maintain the RP4 plasmid as a result of conjugative matings with *E. coli* strain RC709.[13]

These data provide clear evidence that some subsurface environments may have the genetic potential to degrade environmental contaminants. Furthermore, it is possible to introduce specific degradative genetic information into subsurface bacterial communities and monitor its persistence over extended periods of time using modern molecular techniques. However, there is little evidence to suggest that these plasmids or organisms are active under in situ conditions conducive to bioremediation.

BIOLUMINESCENT REPORTER PLASMIDS

Recently, it has been demonstrated that the genes responsible for bioluminescent light emission from certain marine bacteria can be linked directly to degradative genes on plasmids to act as light-emitting "reporters" of degradative gene activity.[14,15] This new bioluminescent reporter can act as a biosensor to measure both the exposure of a bacteria to a specific chemical and the induction of activity of degradative genes.

This new reporter technology was demonstrated using the naphthalene degradative genes of the naphthalene catabolic plasmid NAH7[16] found in some *P. putida* strains. The NAH7 plasmid is evolutionarily closely related in structure and function to the TOL plasmid. On the NAH7 plasmid, the *nah* catabolic genes occupy a relatively small portion of the 83-kb plasmid (Figure 18.1). The *nah* genes are organized in two regulated clusters, or operons. The first operon, which encodes enzymes of the upper pathway, is responsible for the dioxygenase (*nahA*) mediated oxidation of naphthalene to a *cis*-naphthalene dihydrodiol intermediate and eventually to salicylate, the product of *nahF*. The upper pathway operon is composed of a promoter region where the RNA polymerase binds and begins transcription of the *nahA-F* genes to mRNA. The second, or lower pathway, operon is responsible for the oxidation of salicylate, initiated by *nahG*, to central intermediates in the tricarboxylic acid cycle.

Separating the two operons is the regulatory gene *nahR*, which regulates the

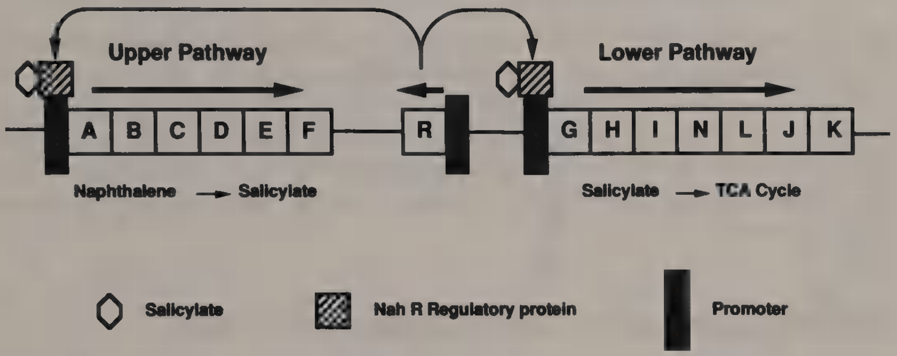
Upper Pathway
Lower Pathway
A
B
C
D
E
F
R
G
H
I
N
L
J
K
Naphthalene → Salicylate
Salicylate → TCA Cycle
Salicylate
Nah R Regulatory protein
Promoter

Figure 18.1. Structure and organization of naphthalene catabolic genes of the NAH7 plasmid.

expression of both pathways. In the absence of naphthalene, the protein product of *nahR* binds near the promoter regions for both the upper and lower pathways and does not allow efficient RNA polymerase binding and transcription.[17] In the presence of naphthalene, a small amount of the metabolite salicylate accumulates and binds to the *nahR* gene product, presumably changing its conformational form and allowing the RNA polymerase to transcribe the genes.[16] This salicylate induction results in a high level of gene expression (transcription) and eventual synthesis of the catabolic enzymes.

Two genetic engineering strategies were used to develop bioluminescent reporter plasmids that would respond to naphthalene exposure or salicylate induction with concomitant production of visible light. These strategies are described in Figure 18.2. In both genetic engineering approaches a promoter-

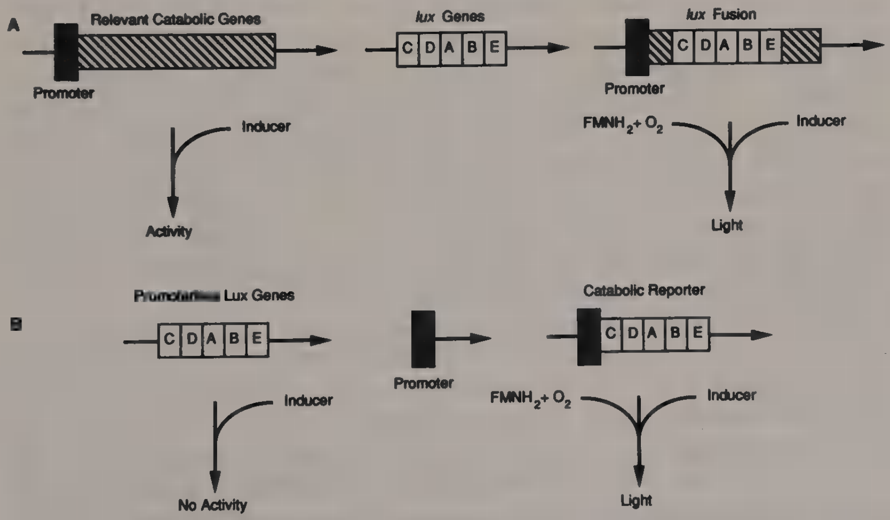
A
Relevant Catabolic Genes
Promoter
lux Genes
C D A B E
lux Fusion
C D A B E
Promoter
Inducer
Activity
$FMNH_2 + O_2$
Inducer
Light
B
Promoterless Lux Genes
C D A B E
Promoter
Catabolic Reporter
C D A B E
Inducer
No Activity
$FMNH_2 + O_2$
Inducer
Light

Figure 18.2. Strategies used in the genetic construction of bioluminescent reporter plasmids.

less *lux* gene cassette,[18] derived from the *lux* genes of the marine bacteria *Vibrio fischeri,* was used as the source of structural genes for bioluminescence. The *lux* genes CDABE encode both the luciferase that oxidizes a long-chain aldehyde to a fatty acid with light production and a reductase that recycles the fatty acid to the aldehyde. In these *lux* cassettes the genes are inactive until they are placed under control of a promoter of either *nah* or *sal* operons of the NAH7 plasmid.

SENSING AND QUANTIFICATION OF BIOLUMINESCENCE

An on-line remote sensing detection system was developed to accurately quantify bioluminescence. The sensing system consists of a commercial photomultiplier digital display unit (Oriel, Model 7070) with a photomultiplier tube (model 77340) connected to a flexible liquid light pipe and collimating beam probe.[14,15] Light output (at 490 nm) is converted into amperes from a photoelectric-induced effect. Sampling frequency and data acquisition are controlled by an IBM PS/2 personal computer using custom software. In comparison to conventional bioluminescence assays, such as autoradiography and scintillation counting, the photomultiplier detection system is rapid, sensitive, and permits both on-line and in situ determinations of bioluminescence.

GENETIC CONSTRUCTION OF REPORTER STRAINS

Two reporter strains were constructed for this study, both of which utilize the *lux* genes of *Vibrio fischeri.* In the first strain, the promoter for the upper pathway of the naphthalene plasmid NAH7 was cloned into the pUC18 plasmid using standard molecular genetic techniques (Figure 18.3). Taking advantage of convenient restriction sites in this construction, the promoter was subcloned on a 2.3-kilobase fragment and inserted into the promoterless *lux* plasmid vector, pUCD615. The resulting plasmid, pUTK9, forms a transcriptional fusion between the *nah* promoter and the *lux* structural genes. This fusion plasmid was introduced into *Pseudomonas putida* strain PB2440, which also contained an intact NAH7 plasmid. NAH7 was necessary to provide the *nahR* regulatory gene, and for the ability to grow on naphthalene. This bioluminescence reporter strain has been designated RB1351.

The second reporter strain was created by transposon mutagenesis of a *P. fluorescens* strain 5R, using the promoterless *lux* transposon Tn4431 (Figure 18.4). Strain 5R is an environmental isolate from a manufactured gas plant (MGP) soil and possesses a NAH7 homologous plasmid, pKA1. Tn4431 was introduced into this strain by conjugation with *E. coli* HB101, which contains the transposon on a transmissible plasmid, pUCD623 (Figure 18.4). The resulting transconjugant 5RL, containing the *lux* recombinant plasmid pUTK21, was selected for the tetracycline resistance marker also on the

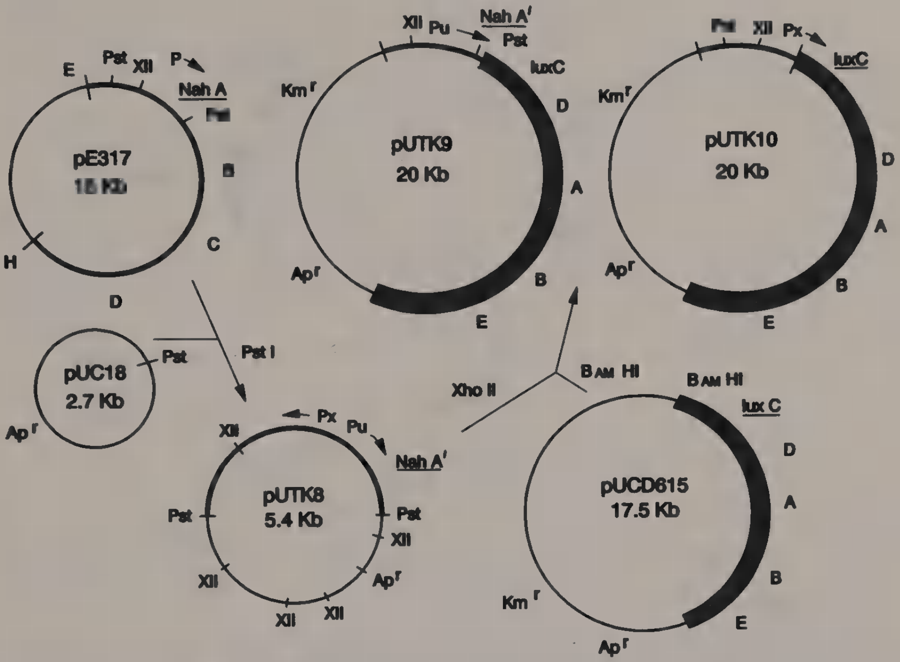
E
Pst
XII
P
Nah A
Pst
pE317
15 Kb
B
C
H
D
pUC18
2.7 Kb
Pst
Ap^r
Pst I
XII
Pu
Nah A'
Pst
Km^r
luxC
D
pUTK9
20 Kb
A
Ap^r
B
E
Pst
XII
Px
luxC
Km^r
pUTK10
20 Kb
D
A
Ap^r
B
E
Px
Pu
XII
Nah A'
pUTK8
5.4 Kb
Pst
Pst
XII
XII
Ap^r
XII
XII
Xho II
BAM HI
BAM HI
lux C
D
pUCD615
17.5 Kb
A
B
Km^r
E
Ap^r

Figure 18.3. Subcloning of the *nah* promoter and construction of pUTK9 and pUTK10. A PstI fragment from pE317 was cloned into the PstI site of pUC18, creating pUTK8. An XhoII fragment of pUTK8 was cloned into the *lux* vector pUCD615 at the BamHI site to create both pUTK9 and pUTK10. P_u, *nah* upper pathway promoter; P_x, promoter in reverse orientation; *XII*, XhoII; *E*, EcoRI; Ap^r, ampicillin resistance; Km^r, kanamycin resistance. Arrows show direction of transcription. From Burlage et al.[14]

Tn4431 and the ability to grow on naphthalene. The transposon was shown to have inserted into the *nahG*, as demonstrated by gene probing with appropriate sequences and by the accumulation of salicylate when supplied with naphthalene. Salicylate hydroxylase is the product of the *nahG* gene, and an interruption in this gene prevents the further degradation of salicylate. In order to avoid the accumulation of salicylate, which is the inducer molecular for both the upper and lower pathways, the recombinant plasmid pUTK21 was moved to another environmental *P. fluorescens* strain that was able to degrade salicylate but not naphthalene. The resulting strain, HK44, is able to completely mineralize naphthalene and exhibits the same bioluminescent characteristics as strain 5RL.

LIGHT EMISSION AFTER NAPHTHALENE INDUCTION

In order to determine whether the *lux* constructions described above are responsive to induction by naphthalene, the following experiment was per-

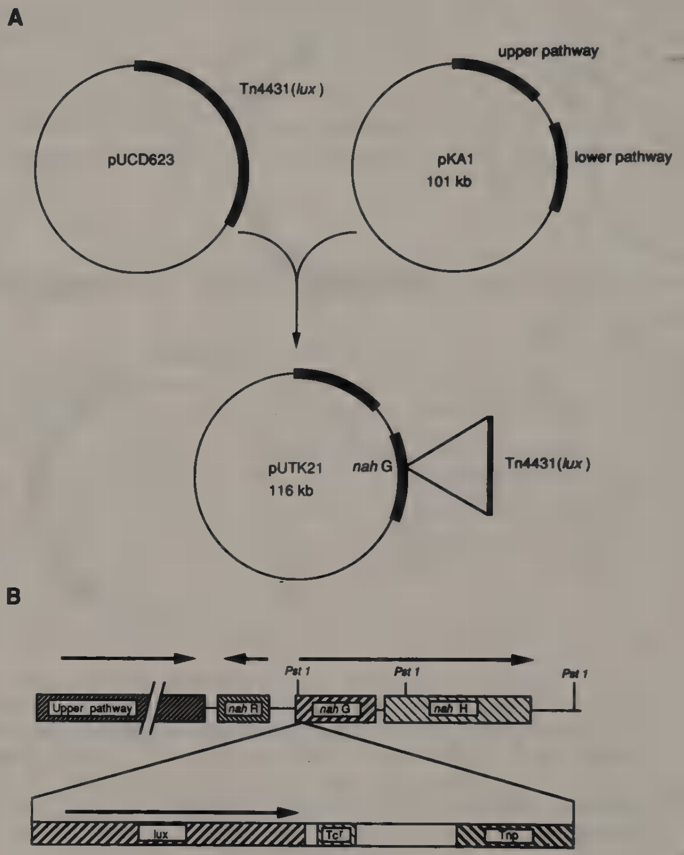
A
upper pathway
Tn4431(*lux*)
pUCD623
pKA1
101 kb
lower pathway
pUTK21
116 kb
nah G
Tn4431(*lux*)
B
Pst 1
Pst 1
Pst 1
Upper pathway
nah R
nah G
nah H
lux
Tc^r
Tnp

Figure 18.4. Construction of bioluminescent reporter plasmid pUTK21 *(A)* and proposed location of lux transposon *(B)*. From King et al.[15]

formed. Colonies of RB1351 were grown on rich agar medium with antibiotic selection. The colonies were exposed to naphthalene vapor by placing naphthalene crystals on the lid of the plates. Figure 18.5 demonstrates the light output of this strain before and after induction. Light induction is fairly rapid (less than 10 min for maximum response) and very stable for many hours under these conditions. The approximately 20-fold increase in light output compares favorably with the results of other investigators who have studied the expression of the upper pathway operon of NAH7. In the original NAH7 host strain, naphthalene induction results in a 20-fold increase in specific mRNA quantity.[19] Results with the HK44 strain are comparable in both magnitude and response. Control experiments using strains without the *nahR* regulatory gene or without the *nah-lux* fusion would not emit light under any conditions, demonstrating the stringent control of this system (data not shown).

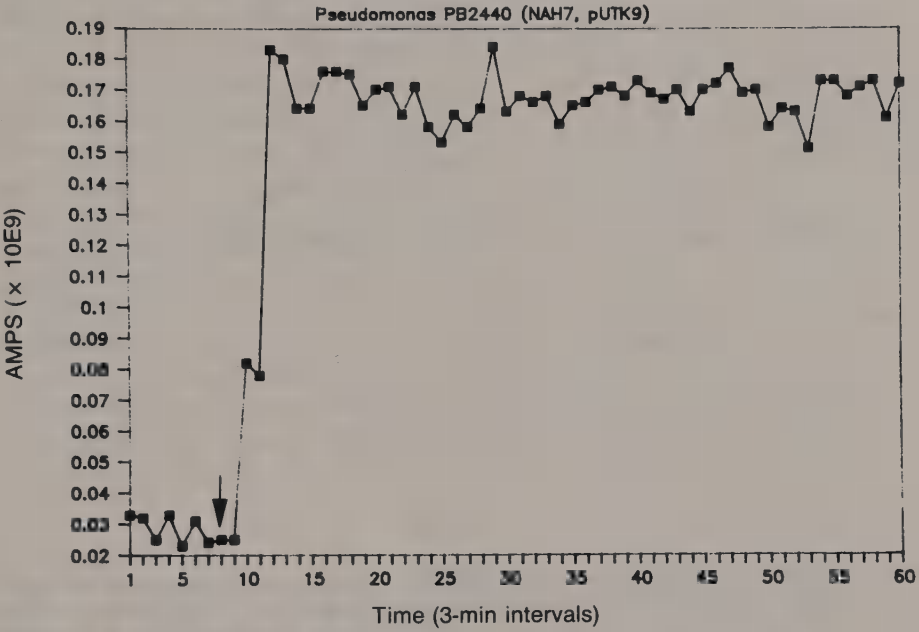

Figure 18.5. Response of RB1351 colonies to naphthalene. *Pseudomonas* RB1351 was grown to mature colonies on LB agar medium. Naphthalene was supplied as vapor from crystals placed on the lid of an inverted plate at time point 8 *(arrow)*. From Burlage et al.[14]

NAPHTHALENE DEGRADATION IS CORRELATED WITH LIGHT PRODUCTION

In order for this strain to qualify as a reporter of genetic expression, it was necessary to correlate the signal (light production) with the relevant phenotype (in this case, naphthalene utilization). This was accomplished with a series of mineralization experiments in which a ^{14}C-labeled naphthalene was added to a culture of RB1351 and then periodically examined for by-products of catabolism. Figure 18.6 shows the course of the catabolic reactions over a period of 3 hr, including the light output during this time. A minimal salts medium was used when naphthalene was the sole carbon and energy source. Under these conditions the growth rate was very slow, and the viable count was unchanged throughout the experiment. As shown in the figure, the degradation of naphthalene proceeds quickly after addition to the culture medium. Light generation also responds rapidly, demonstrating that the promoters controlling light expression and naphthalene degradation are regulated in an identical manner. Subsequent experiments using other media demonstrated that naphthalene utilization was always accompanied by light production, so the responses are correlated.

It was expected that addition of naphthalene to a growing culture in a liquid medium would also result in a rapid bioluminescent response, similar to that

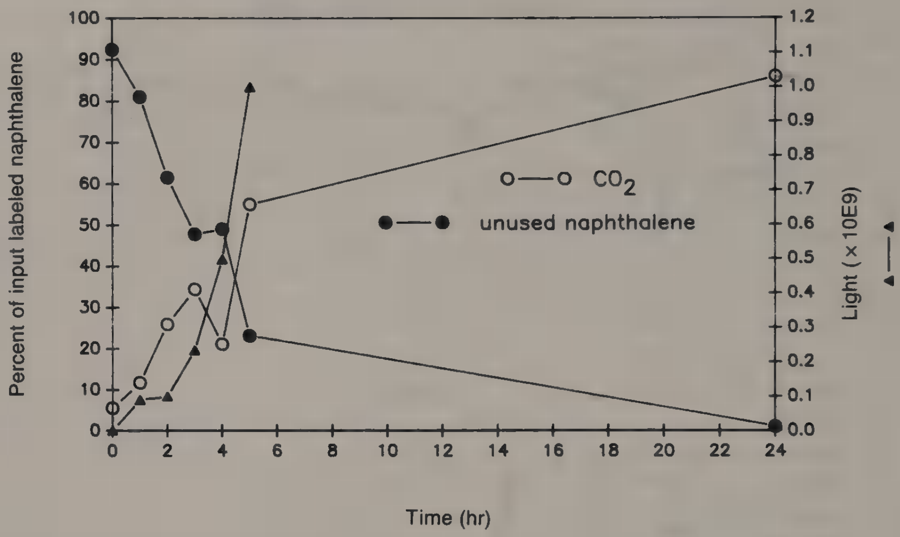

Figure 18.6. Mineralization assay with RB1351. Duplicate vials were incubated with ^{14}C-naphthalene in Basal Salts medium. Fractions of ^{14}C in CO_2 and in unused naphthalene are indicated. Light output was measured immediately before fraction analysis. Cell concentration remained at 3 × 10^7 throughout the experiment. From Burlage et al.[14]

seen with the colonies described above (Figure 18.5). This was not true. In rich medium, naphthalene crystals were added when the culture was in an exponential rate of growth (log phase). Light generation was approximately 4 hr, until the culture had entered a slower rate of growth, and did not become stable until a further 6 hr had elapsed. Mineralization experiments demonstrated that significant naphthalene utilization was not observed during the exponential phase of growth, but increased substantially during the slower rate of growth.[14] This was an unexpected result and suggests that either a catabolite repression is involved in regulation of this operon, or that the operon is growth-rate regulated. This work shows the potential of the *lux* system for describing genetic expression in a unique and useful manner. Further experiments to define this effect are in progress in this laboratory. The results of these experiments will undoubtedly offer greater insight into the ways in which degradative strains may be utilized for the destruction of contaminants in a bioreactor setup.

DYNAMIC SENSING OF NAPHTHALENE DEGRADATION AND LIGHT OUTPUT

A continuous culture system was employed to determine the dynamic response nature of the bioluminescent reporter strains to periodic naphthalene exposure. The experimental system has been previously described[20] and consists of a 1-L reactor supplied with a dual nutrient feed supply either saturated with naphthalene or containing no naphthalene. An on-line offgas sampling system was used to determine the naphthalene concentration in the gas phase, and the liquid naphthalene concentration in the reactor was calculated using Henry's law.[21] The response of strain HK44 to square wave perturbations in naphthalene exposure with a frequency of 4 hr is shown in Figure 18.7. A 15-min lag was observed in the bioluminescent response to changes in naphthalene exposure. Bioluminescence increased linearly at a rate of 0.39 μamps/hr during the 2-hr naphthalene exposure period, followed by a near-linear reduction in light output during the 2-hr period with no naphthalene. During the naphthalene exposure phase of the feed cycle, the liquid naphthalene concentration in the chemostat increased to a steady-state concentration of between 0.4–0.5 mg/L, and the naphthalene degradation rate remained almost constant. When naphthalene addition to the reactor was ceased, the reactor liquid naphthalene concentration decreased exponentially to 0.1 mg/L. Interestingly,

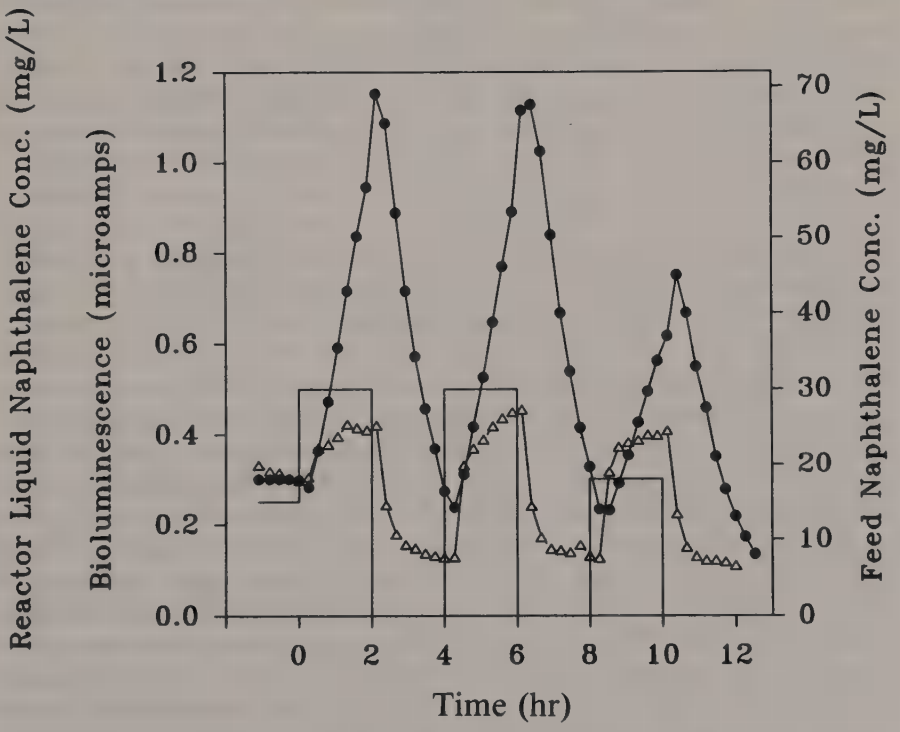

Figure 18.7. Dynamic response of strain HK44 in continuous culture to 4 hr square-wave perturbations in naphthalene. Chemostat operating conditions were as follows: temperature, 25°C; dilution rate, 0.4 hr^{-1}; dual feed system containing a minimal salts medium supplemented with sodium succinate (100 mg/L), yeast extract (100 mg/L), tetracycline (14 mg/L), and naphthalene (to one feed supply only, 30 mg/L). ●, bioluminescence; ———, naphthalene feed concentration; △, reactor liquid naphthalene concentration. From King et al.[15]

in the third perturbation cycle when the feed naphthalene concentration was reduced from 30 to 18 mg, both the rate of increase in bioluminescence (0.26 μamps/hr) and the peak bioluminescence attained were reduced. These data suggest that the rate of increase in bioluminescence may be proportional to the naphthalene degradation rate. By using square wave perturbations ranging in periodicity from 0.5 to 16 hr, bioluminescence was shown to be related to naphthalene exposure. During prolonged continuous naphthalene exposure under steady-state conditions, the level of bioluminescence remained constant.

IN SITU SIMULATIONS

An important application of the bioluminescent reporter technology is for in situ analysis of microbial biodegradative activity. In comparison to conventional activity assays, bioluminescent assays are noninvasive, nondestructive, rapid, and population specific. We have already demonstrated bioluminescence sensing in a chemostat, which may be considered analogous to a waste treatment reactor. However, an alternative application for the technology is for monitoring activity in situ in soils or groundwaters. Remote sensing of bioluminescence in such complex matrices is inherently more problematical than it is in liquid culture systems. Quenching of light by the opaque matrix, particulate interference, and the development of biofilms might all be expected to affect sensitivity and light monitoring. To demonstrate the utility of the bioluminescent reporter technology in such complex matrices, experiments were conducted using stirred batch soil slurries of contaminated and uncontaminated soils. Slurries were prepared by the addition of 10 mL of uninduced HK44 cells (10^9 cells/mL) in phosphate buffered saline to 10 g of soil. The slurries were stirred to provide aeration, and bioluminescence was monitored by placing the liquid light pipe probe into the slurry. Two soil types were used: an uncontaminated Etowah soil obtained from East Tennessee and a soil from an MGP site contaminated with naphthalene (1240 mg/kg) and other polycyclic aromatic hydrocarbons (total PAH concentration, 3227 mg/kg). As expected, no bioluminescence was observed in the uncontaminated Etowah soil used as a control (Figure 18.8). However, bioluminescence indicative of naphthalene exposure and degradation was detected in the MGP soil naturally contaminated with PAH, and when exogenous naphthalene (10 mg) was added to the Etowah control soil (Figure 18.8). Light production in the MGP soil is significant, since it was induced by endogenous naphthalene and this illustrates a further use of such reporter strains, namely, as an assay for the bioavailability of contaminants in environmental matrices.

Additional experiments would be required to correlate the bioluminescence levels reported in Figure 18.8 with actual biodegradative activity levels. In this respect it would be necessary, for example, to conduct various calibration experiments using constitutive light-producing strains in the different soils to

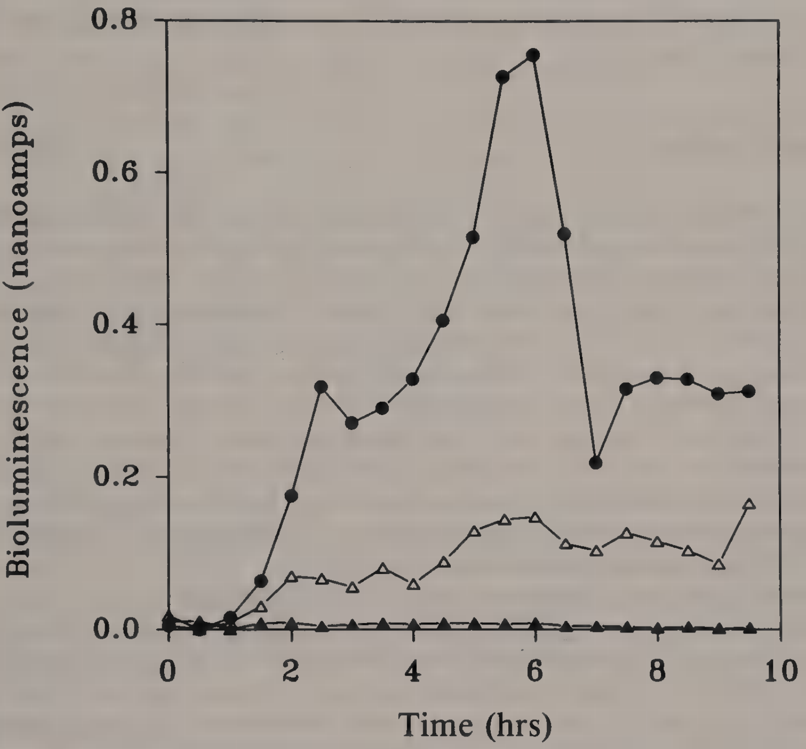

Figure 18.8. Detection of bioluminescence in stirred batch soil slurries inoculated with uninduced cells of strain HK44. The slurry consisted of 10 g soil and 10 mL of cell suspension (10^9 cells/mL). Etowah soil (uncontaminated), ▲; Etowah soil amended with 10 mg naphthalene, △; MGP soil, ●. From King et al.[15]

determine the relative light-quenching effects of the respective soil matrices. Then from the results of pure culture chemostat studies, it should be possible to convert the in situ response data into actual activity levels. However, the results reported here can be used as a qualitative indicator of activity, and it is possible to hypothesize about the differences observed in the bioluminescent responses of strain HK44 in the two soil types. The rate of increase in light production was greater in the MGP soil than in the Etowah soil amended with exogenous naphthalene, possibly indicating a higher initial biodegradative activity in the former. A number of factors could explain the subsequent transient peak in activity in the MGP soil—for example, exhaustion of bioavailable naphthalene (the inducer of bioluminescence) or a decrease in the survival of the introduced reporter strain. It was hoped that the latter effect could be minimized by using strain HK44, which was originally isolated from an MGP soil. Although it was not performed in these studies, the survival of the bioluminescent reporter strain could be followed by DNA hybridization procedures. In the Etowah soil amended with naphthalene, bioluminescence—

and hence naphthalene biodegradation activity—remained relatively constant for up to 16 hr.

CONCLUSIONS

The successful use of microbial biodegradation processes for in situ remediation of subsurface contamination will require further development of strategies to control the structure and activities of microbial communities. These controls can be used to modulate the activities of indigenous subsurface microorganisms, if they exhibit the genetic potential or physiological capacity for contaminant degradation. Although degradative capacity exists in some surface environments, it may be necessary to enhance such populations or to add nonindigenous organisms to the environment. Based on simulations in the laboratory, bacteria containing degradative (catabolic) plasmids can be introduced and maintained in subsurface materials. Additionally, plasmids may also be introduced to indigenous subsurface microorganisms to deliver catabolic genes into the microbial community.

Regardless of the genetic capacity of subsurface microbial communities for biodegradation, there are still questions concerning maintenance of the functional activity of the organisms in the environment. These questions concern the need to deliver sufficient carbon and energy sources as well as electron acceptors to maintain biochemical activity. There is also the need to develop reliable methods to measure the catabolic activity and response of the degradative organisms to environmental manipulation in remediation practice.

The applications of genetic engineering techniques to develop reporters of biological activity may contribute to the analysis of functional activity in the environment. The development of bioluminescent reporters for catabolic activity in naphthalene degradation is an example of such applications of genetic engineering methods. The *lux* reporter strains developed for sensing degradation have been shown to be stable in batch and continuous culture simulations of environmental treatment. Using remote light sensing techniques, light emission from as few as 10^6 organisms per gram of soil can be detected during naphthalene degradation. The threshold of light detection with current light measuring systems is as low as 10^3 bioluminescent bacteria. It has been demonstrated that light emission is directly correlated with naphthalene degradation. The induction of the light response of the reporter strains is also rapid, occurring in as little as 15 min after exposure to naphthalene. Since light emission is a process that is coupled to intracellular accumulation of naphthalene and biochemical induction of naphthalene degradative genes as well as the *lux* genes, light production is also a direct measure of bioavailability of naphthalene and possibly of other related compounds in environmental matrices. Preliminary evidence has recently been obtained showing that phenanthrene also induces a bioluminescent response (unpublished results).

This exposure to, and bioavailability of, indigenous naphthalene contaminants in MGP soils was clearly demonstrated by addition of the reporter strains to the naturally contaminated soils and the induction of light by this very specific bioassay method.

It is anticipated that such reporter strain technology will find significant use in establishing environmental regimes most conducive to microbial degradative activity, hence bioremediation. In addition, the reporter strain technology may also provide a practical measure of the presence of and exposure to specific chemical contaminants in the subsurface environment. In this regard, the technology will likely have joint use in contributing to optimization of remediation practices as well as environmental toxicological applications in exposure assessment.

ACKNOWLEDGMENTS

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List of Authors

Gina Abbate, Department of Biology, University of Dayton, 300 College Park Drive, Dayton, Ohio 45469–2320

Neal Adrian, The University of Oklahoma, Department of Botany and Microbiology, 770 Van Vleet Oval, Norman, Oklahoma 73019–0245

Robert Burlage, Center for Environmental Biotechnology, Department of Microbiology and the Graduate Program in Ecology, The University of Tennessee, Knoxville, Tennessee 37932

Carl E. Cerniglia, Microbiology Division, National Center for Toxicological Research, Food and Drug Administration, Jefferson, Arkansas 72079

Rita R. Colwell, University of Maryland, Center of Marine Biotechnology, 600 East Lombard Street, Baltimore, Maryland 21202

Miriam Diamond, Institute for Environmental Studies, University of Toronto, Toronto, Ontario M5S 1A4

Elizabeth Edwards, Stanford University, Department of Civil Engineering, Stanford, California 94305–4020

Robert P. Eganhouse, Southern California Coastal Water Research Project, 646 W. Pacific Coast Highway, Long Beach, California 90806

William Eng, Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996

Hayla E. Evans, Roda Environmental Research, P.O. Box 447, Lakefield, Ontario K0L 2H0

E. Michael Godsy, Stanford University, Department of Civil Engineering, Stanford, California 94305–4020

Richard W. Gossett, Southern California Coastal Water Research Project, 646 W. Pacific Coast Highway, Long Beach, California 90806

Dunja Grbic-Galic, Stanford University, Department of Civil Engineering, Stanford, California 94305–4020

Dorothy D. Hale, Technology Applications, Inc., Environmental Research Laboratory, USEPA, Athens, GA 30613–0801 and Department of Microbiology, University of Georgia, Athens, Georgia 30602

Susan M. Henry, Environmental Sciences Division, Lawrence Livermore National Laboratory, P.O. Box 5507, L-453, Livermore, California 94550

J. M. Henry King, Center for Environmental Biotechnology, Department of Microbiology and the Graduate Program in Ecology, The University of Tennessee, Knoxville, Tennessee 37932

Ivor T. Knight, Department of Biology, James Madison University, Harrisonburg, Virginia 22807

Jussi Kukkonen, University of Joensuu, Department of Biology, P.O.Box 111, SF-80101 Joensuu, Finland

James L. Lake, U.S. Environmental Protection Agency, Environmental Research Laboratory-Narragansett, 27 Tarzwell Drive, Narragansett, Rhode Island 02882

Peter F. Landrum, Great Lakes Research Laboratory, National Oceanographic and Atmospheric Administration, Ann Arbor, Michigan 48105

Frank Larimer, Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831

Henry Lee II, Pacific Ecosystem Branch, Environmental Research Laboratory-Narragansett, U.S. Environmental Protection Agency, Marine Science Center, Newport, Oregon 97365

Donald Mackay, Institute for Environmental Studies, University of Toronto, Toronto, Ontario M5S 1A4

Kevin P. Mayer, U.S. Environmental Protection Agency, Region IX, 75 Hawthorne Street, H-6–4, San Francisco, California 94105

John F. McCarthy, Environmental Sciences Division, Oak Ridge National Laboratory, Building 1505, M.S. 6036, P.O. Box 2008, Oak Ridge, Tennessee 37831–6036

Alan J. Mearns, U.S. National Oceanic and Atmospheric Administration, Ocean Assessments Division-Pacific Office, 7600 Sand Point Way N.E., Seattle, Washington 98115

Aimo Oikari, University of Joensuu, Department of Biology, P.O.Box 111, SF-80101 Joensuu, Finland

Frank A. Osterman, U.S. Environmental Protection Agency, Environmental Research Laboratory-Narragansett, 27 Tarzwell Drive, Narragansett, Rhode Island 02882

Anthony V. Palumbo, Environmental Sciences Division, Oak Ridge National Laboratory, P. O. Box 2008, Oak Ridge, Tennessee 37831

James R. Pratt, School of Forest Resources and Graduate Program in Ecology, Penn State University, University Park, Pennsylvania 16802

Richard J. Pruell, U.S. Environmental Protection Agency, Environmental Research Laboratory-Narragansett, 27 Tarzwell Drive, Narragansett, Rhode Island 02882

K. Ramanand, The University of Oklahoma, Department of Botany and Microbiology, 770 Van Vleet Oval, Norman, Oklahoma 73019–0245

John E. Rogers, Environmental Research Laboratory, USEPA, Athens, GA, 30613–0801, and Department of Microbiology, University of Georgia, Athens, Georgia 30602

Gary S. Sayler, Center for Environmental Biotechnology, Department of Microbiology and the Graduate Program in Ecology, The University of Tennessee, Knoxville, Tennessee 37932

Scott D. Schermerhorn, Department of Biology, University of Dayton, 300 College Park Drive, Dayton, Ohio 45469–2320

Paul M. Sherblom, Mote Marine Laboratory, 1600 Thompson Parkway, Sarasota, FL 34236 (NOTE: Work reported in Chapter 9 was performed when Paul Sherblom was associated with the Environmental Sciences Program, University of Massachusetts at Boston, Boston, MA 02125)

A. Södergren, Department of Ecology, Chemical Ecology/Ecotoxicology, Lund University, S-223 62 Lund, Sweden

Warren Stiver, Institute for Environmental Studies, University of Toronto, Toronto, Ontario M5S 1A4

Gerald W. Strandberg, Chemical Technology Division, Oak Ridge National Laboratory, P. O. Box 2008, Oak Ridge, Tennessee 37831–6227

Joseph M. Suflita, The University of Oklahoma, Department of Botany and Microbiology, 770 Van Vleet Oval, Norman, Oklahoma 73019–0245

Roy M. Ventullo, Department of Biology, University of Dayton, 300 College Park Drive, Dayton, Ohio 45469-2320

Juergen Wiegel, Department of Microbiology, University of Georgia, Athens, Georgia 30602

David R. Young, U.S. Environmental Protection Agency, Environmental Research Laboratory-Narragansett, Pacific Ecosystems Branch, Hatfield Marine Science Center, Newport, Oregon 97365

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